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Insights into Cellular Stress Revealed by SIRT7

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Insights into Cellular Stress Revealed by SIRT7

By

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Abstract

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Aging was once thought to be an inexorable process, driven by wear and tear that steadily accumulates in cells. However, we have now come to the realization that aging is subject to modulation by genetic pathways, including the insulin/IGF-1 pathway, the mTOR pathway, and sirtuins. A commonality among these pathways is that they exert their pro-longevity and pro-health effects through responding to stressors, such as diet, oxidative stress, or protein-folding stress, and initiating a self-protective program in the cell. Sirtuins have been shown to play a major role in the cellular response to calorie restriction and oxidative stress. However, their role in protein homeostasis and in particular, the response to stress from unfolded proteins has not been well-characterized. We explored this topic by studying the role of SIRT7, a nuclear member of the mammalian sirtuin family, in responding to protein-folding stress in the endoplasmic reticulum (ER) and in mitochondria. We found that SIRT7 plays a pivotal role in the response to ER stress and mitochondrial stress by inhibiting the transcription of ribosomal subunits and mitochondrial ribosomal subunits, which results in a reduction in translation, allowing more time for proper protein folding. We found that SIRT7 mediates its effects by binding to the transcription factors Myc and NRF-1, which are master regulators of ribosomal subunits and mitochondrial biogenesis respectively. Our study suggests that the stress response mediated by SIRT7 at the cellular level may have important implications for obesity-associated diseases and hematopoietic stem cell function.

Dedicated to...

Mom and Dad, for your inexhaustible love and support…

Yutong, may you never age…

and Sandy, for filling my life with fun and warmth

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Chapter 1

An Introduction to Aging, Stress, and Sirtuins

Nothing seems as inexorable as the aging process. From the moment of our births, the sands of time relentlessly pour down in the hourglass from the time we have left to the time that we have already exhausted. Aside from freak accidents or lifestyle decisions, it seems that there is very little we can do about the aging process. Besides general changes in appearance, aging also leads to an exponential increase in the incidence of cancer, degenerative and inflammatory diseases such as arthritis, osteoporosis, cardiovascular disease, and Alzheimer's, and metabolic diseases such as diabetes (Campisi, 2013). As these diseases can have a major impact on quality of life, health span, the period of life free from serious chronic diseases, is just as important as lifespan in considering aging. One of the major discoveries in recent years is that the rate of aging is not as set in stone as previously thought. Instead, aging is influenced at the genetic level, and the activities of the genes that regulate aging can be modulated to influence not only lifespan, but also health span.

The Genetic Regulators of Aging

The first intervention that was shown to slow the aging process is calorie restriction (CR), consisting of a diet with a 30-40% reduction in caloric intake. Studies in the 1930s on mice fed a CR diet led to the surprising result that the mice actually lived longer than mice fed ad libitum (McCay *et al.*, 1935). The pro-longevity effect of calorie restriction has since been seen in organisms as diverse as worms, flies, and yeast (Kenyon, 2010). Studies performed in primates have not yet definitively shown lifespan extension, but they have found many beneficial effects on health (Colman *et al.*, 2009; Mattison *et al.*, 2012). Overall, it is remarkable that CR leads to not only extended lifespan, but extended health span as well. At first, CR was thought to lead to its beneficial effects by reducing the metabolic rate of organisms and thus, leading to less wear and tear (Sohal and Weindruch, 1996). However, it is now known that CR mediates its effects through multiple genetic pathways, among them the insulin/insulin-like growth factor (IGF-1) pathway, the mechanistic target of rapamycin (mTOR) pathway, and sirtuins (Kenyon, 2010).

Insulin/IGF-1 Signaling

The first genetic regulator of aging was discovered in *C. elegans* with a mutation in *daf-2* that reduces the activity of the gene (Kenyon *et al.*, 1993). Worms harboring the *daf-2* mutation not only lived longer, but were also more youthful for a longer period of time (Kenyon *et al.*, 1993). *daf-2* encodes the *C. elegans* homolog of the IGF-1 receptor (Kimura *et al.*, 1997). Later studies found that the decreased activity of *daf-2* leads to an increase in the activity of *daf-16*,

which is essential for *daf-2*-mediated lifespan extension (Kenyon *et al.*, 1993). *daf-16* was found to encode the *C. elegans* homolog of FOXO, a family of forkhead binding transcription factors that promotes the expressions of numerous genes involved in stress response (Lin *et al.*, 1997; Ogg *et al.*, 1997).

The insulin/IGF-1 receptor functions as a nutrient sensor and its activity is increased by nutrient-rich conditions. The receptor signals to the nucleus via a signal transduction pathway that includes Akt and phosphoinositide-3-kinase (PI3K), ultimately inhibiting the activity of FOXO transcription factors (Tatar *et al.*, 2003). Under nutrient-poor conditions or with the *daf-2* mutation that reduces the activity of the receptor, FOXO becomes less inhibited and leads to the transcriptions of stress resistance genes that are thought to contribute to lifespan extension (Lin *et al.*, 1997; Ogg *et al.*, 1997). Through a FOXO-independent mechanism, inhibition of insulin/IGF-1 signaling also leads to an increase in autophagy, which is the cell's way of recycling its components and in essence, refreshing itself. Autophagy has been shown to be essential to insulin/IGF-1 related lifespan extension (Melendez *et al.*, 2003)..

Since the discovery of the connection between insulin/IGF-1 receptor signaling and lifespan in *C. elegans*, other studies have shown that reduced signaling from the receptor or downstream components in the pathway is associated with increased lifespan in flies, mice, and dogs (Bartke, 2008; Yuan *et al.*, 2009; Kappeler *et al.*, 2008; Selman *et al.*, 2008; Greer *et al.*, 2011). The connection between the activity of FOXO transcription factors and lifespan has also been shown in numerous organisms and certain alleles of FOXO have been strongly correlated with long lifespan in various groups of humans (Wilcox *et al.*, 2008; Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009).

mTOR

Another genetic pathway that has been strongly implicated in regulating lifespan is mTOR, which is a kinase that senses the nutrient status of the cell and coordinates multiple cellular processes, including growth, translation, and autophagy, among others (Laplante and Sabatini, 2012). When the activity of mTOR is inhibited, such as through application of rapamycin or through exposure to a low nutrient environment in CR, the cell switches from an anabolic state of growth to a state of self-preservation and stress resistance (Laplante and Sabatini, 2012).

A key component of how mTOR affects lifespan is thought to be through its regulation of translation through 4E-BP, an inhibitor of translation, and ribosomal S6 kinase (S6K), which promotes translation (Kaeberlein and Kennedy, 2011). When mTOR is inhibited, S6K activity decreases, and deficiency in S6K and its homologs has been shown to result in lifespan extension in yeast, worms, flies, and mice (Fabrizio *et al.*, 2001, Kapahi *et al.*, 2004, Kaeberlein *et al.*, 2005, Hansen *et al.*, 2007, Pan *et al.*, 2007, Selman *et al.*, 2009). Mechanistically, inhibition of S6K activates adenosine monophosphate

kinase (AMPK) and results in a metabolic state that is reminiscent of CR (Kaeberlein and Kennedy, 2011). Inhibition of mTOR also leads to an upregulation of autophagy, which as in insulin/IGF-1 signaling, is an important component contributing to lifespan extension (Kenyon, 2010).

The Role of Sirtuins in Lifespan Extension and Stress Resistance

The sirtuins are an evolutionarily conserved family of NAD+-dependent deacetylases (Finkel *et al.*, 2009). The founding member of the family, Sir2, was discovered in yeast and shown to effect lifespan extension when overexpressed (Kaeberlein *et al.*, 1999) and was shown to be required for CR-mediated lifespan extension in yeast (Lin *et al.*, 2000). Homologues of Sir2 have since been discovered in worms, flies, and mammals and overexpression of the homologues has led to lifespan extension in both *D. melanogaster* (Rogina and Helfand, 2004) and *C. elegans* (Tissenbaum and Guarente, 2001). In mammals, there are seven members of the sirtuin family (SIRT1-7) localized in various cellular compartments (Haigis and Guarente, 2006). So far, only one mammalian sirtuin, SIRT6, has been shown to promote lifespan extension when overexpressed (Kanfi *et al.* 2012). However, mammalian sirtuins have been shown to mediate essential aspects of calorie restriction (Chen *et al.*, 2005; Qiu *et al.*, 2010; Someya *et al.*, 2010; Guarente, 2013) and protect against various cellular stresses, such as genomic instability (Bosch-Presegue and Vaquero, 2013), oxidative stress (Merksamer *et al.*, 2013), and physiological damage induced by high fat diets (Pfluger *et al.*, 2008). In the following sections, latest findings on how sirtuins respond to each of these stresses will be reviewed.

Genomic Instability

Preserving the genomic material at the heart of the cell is an essential process, disruption of which can lead to cell death, senescence, or tumorigenesis (Aguilera and Gomez-Gonzalez, 2008). Many members of the sirtuin family have been shown to play a protective role in this process. The first study showing the role of sirtuins in protecting against genomic instability was performed in *S. cerevisiae*, where Sir2 was shown to silence the rDNA loci and prevent the formation of extrachromosomal rDNA circles (ERCs) (Kaeberlein *et al.*, 1999). ERCs had been shown to promote aging in *S. cerevisiae* by hijacking the cellular machinery for DNA replication and repair, resulting in genomic instability (Sinclair and Guarente, 1997). By inhibiting the formation of ERCs, Sir2 is able to extend lifespan (Kaeberlein *et al.*, 1999).

Most of the mammalian sirtuin family members have been shown to play a protective role against genomic instability (Bosch-Presegue and Vaquero *et al.*, 2013). SIRT1 is a nuclear sirtuin that is the closest homolog to yeast Sir2 (Finkel *et al.,* 2009). It has been shown to promote DNA repair by modulating the activity of the DNA repair protein NBS1, and SIRT1-deficient cells have delayed cell cycle progression upon exposure to ionizing radiation (Yuan *et al.*, 2007). In

addition, SIRT1 has been shown to localize to double-stranded DNA breaks (DSBs), where it is essential for DNA repair (Oberdoerffer *et al.*, 2008). Lastly, SIRT1 knockout (KO) mice are embryonic lethal, succumbing to chromosomal abnormalities and impaired DNA repair (Wang *et al.*, 2008). More generally, SIRT1 has been shown to promote the spread of heterochromatin, leading to silencing of genes and protection from DNA damage (Vaquero *et al.*, 2004, Vaquero *et al.*, 2007).

SIRT2 is a predominantly cytoplasmic protein that has also been demonstrated to play a role in combating genomic instability (Bosch-Presegue and Vaquero *et al.*, 2013). Mouse embryonic fibroblasts (MEFs) derived from SIRT2 KO mice show increased centrosome amplification and defects in mitotic progression due to SIRT2's role in promoting the function of the anaphase promoting complex/cyclosome (APC/C) (Kim *et al.*, 2011). A different study showed that SIRT2 is essential for modulation of chromatin in response to mitotic stress and is important for S phase progression (Serrano *et al.* 2013). SIRT2 KO mice in both studies were found to have genomic instability that promoted tumorigenesis (Kim *et al.*, 2011, Serrano *et al.*, 2013).

Of the three mitochondrial sirtuins, SIRT3 and SIRT4 have been shown to play a role in genomic stability by regulating mitochondrial function (Kim *et al.*, 2010, Jeong *et al.*, 2013). SIRT3's role in protecting against genomic instability is mainly through suppression of oxidative stress, which will be discussed later. In brief, SIRT3 deacetylates and increases the activity of superoxide dismutase 2 (SOD2 or Mn-SOD), which protects against oxidative stress-induced genomic instability by scavenging for reactive oxygen species (ROS), which have been shown to directly damage DNA bases (Kim *et al.*, 2010). SIRT4 is required for DNA damage-mediated blockage of glutamine metabolism (Jeong *et al.*, 2013). In SIRT4-deficient cells exposed to DNA damage, there was an impaired DNA damage response and a greater incidence of aneuploidy (Kim *et al.*, 2013). So far, SIRT5, the third mitochondrial sirtuin, has not been found to influence genomic stability, but it is among the least studied of the sirtuins.

Numerous studies have shown that SIRT6, a nuclear sirtuin, plays an extensive role in promoting DNA damage repair and suppressing genomic instability. SIRT6 KO cells were found to have a defect in base excision repair (BER), while SIRT6 KO mice died 2-3 weeks after birth from premature aging symptoms, possibly brought about by the defect in DNA repair (Mostoslavsky *et al.*, 2006). SIRT6 has been shown to be one of the earliest proteins recruited to DBSs, where it remodels chromatin at the beginning of the DNA repair process (Toiber *et al.*, 2013) and promotes DNA end resection (Kaidi *et al.*, 2010). SIRT6 has also been shown to respond to oxidative stress-induced DSBs, where in conjunction with poly[adenosine diphosphate(ADP)-ribose] polymerase 1 (PARP1), it stimulates both homologous recombination (HR) and non-homologous end joining (NHEJ) to repair the damage (Mao *et al.*, 2011). Lastly, SIRT6 was shown to associate with telomeric chromatin, where it allows for the

stable association of WRN, a protein whose mutation has been linked to the premature aging disease Werner's syndrome (Michishita *et al.*, 2008). Absence of SIRT6 leads to abnormal telomere structure and an increase in end-to-end chromosomal fusions and cellular senescence (Michishita *et al.*, 2008).

SIRT7, like SIRT1 and SIRT6, is a nuclear sirtuin with preferential localization to the nucleolus (Ford *et al.*, 2006). While SIRT7's role in DNA repair has not been extensively characterized, some studies have hinted at a possible role in DNA repair. Its overexpression has been shown to increase the efficiency of NHEJ and HR, but the mechanism is unknown (Mao *et al.*, 2011). In a proteomic study, SIRT7 has been shown to interact with Rap1-interacting factor 1 homolog (RIF1) (Tsai *et al.*, 2012). RIF1 is known to localize to DNA breaks and participate in ATM-mediated DNA repair (Silverman *et al.*, 2004). Finally, a study has shown that SIRT7-deficient cardiomyocytes show increased sensitivity to oxidative and genotoxic stress, and SIRT7 KO mice develop cardiomyopathy (Vakhrusheva *et al.*, 2008). While the links between SIRT7 and promotion of genomic integrity are tantalizing, there is no study at the current time that has shown a clear mechanistic link.

The prominent role played by sirtuins in protecting against genotoxic stress is demonstrated by the embryonic lethality of SIRT1 KO mice and the drastically shortened lifespan of SIRT6 KO mice (Wang *et al.*, 2008, Mostoslavsky *et al.*, 2006). Meanwhile, SIRT1 heterozygous (Wang *et al.*, 2008) and conditional SIRT6 KO mice (Sebastian *et al.*, 2012) are more prone to tumorigenesis. Additionally, the knockout mice for SIRT2 (Kim *et al.*, 2011), SIRT3 (Kim *et al.*, 2010), and SIRT4 (Jeong *et al.*, 2013) are all more prone to cancer development. Thus, while only SIRT6 has been shown to extend lifespan, many mammalian sirtuins play an important role in maintaining genomic integrity and maintaining health.

Oxidative Stress

Mitochondria are the powerhouses of eukaryotic cells, but they come with the caveat of ROS production, a byproduct of using oxygen to power oxidative phosphorylation. ROS include the hydroxyl radical, hydrogen peroxide, and the superoxide anion, among others. These can level damage against essential cellular components such as DNA, proteins, and lipids (Merksamer *et al.,* 2013). Originally, the mechanism through which CR was thought to extend lifespan was via a reduction in ROS due to a decrease in metabolic rate and mitochondrial activity (Sohal and Weindruch, 1996). However, it was later found that instead of decreasing mitochondrial activity, CR actually promotes it (Nisoli *et al.*, 2005), which deepened the mystery of how CR lowers ROS. We now know that sirtuins, and namely SIRT3, are the key to this mystery.

SIRT3 regulates global acetylation levels in the mitochondria (Lombard *et al.*, 2007). Two separate studies found that in mice, reduction of ROS during CR requires the presence of SIRT3 (Qiu *et al.*, 2010; Someya *et al.*, 2010). The

studies found that SIRT3 reduced ROS by increasing the activity of superoxide dismutase 2 (SOD2), which scavenges for ROS, (Qiu *et al.*, 2010) and by increasing the activity of isocitrate dehydrogenase 2 (Idh2), which increases the mitochondrial ratio of reduced to oxidized glutathione (Someya *et al.*, 2010). Two other studies also showed that SIRT3 reduced ROS via SOD2 (Tao *et al.*, 2010, Kim *et al.*, 2010). The physiological relevance of SIRT3's ability to combat oxidative stress was demonstrated by its requirement for combating hearing loss during CR and its ability to rejuvenate aged hematopoietic stem cells (Brown *et al.*, 2013). Studies have also found that SIRT3's ability to function as a tumor suppressor is dependent on its ability to prevent ROS-mediated genotoxic damage (Kim *et al.*, 2010; Bell *et al.*, 2011). Lastly, SIRT3 has also been shown to decrease ROS production by modulating the activity of the mitochondrial complexes (Bell and Guarente, 2011).

Besides SIRT3, SIRT1 is the other mammalian sirtuin that has been shown to play a major role in combating oxidative stress. Unlike SIRT3, which directly acts on mitochondrial enzymes and components to reduce ROS production, SIRT1 functions at the transcriptional level to induce the expression of stress resistance genes. In response to oxidative stress, SIRT1 forms a complex with the transcription factor FOXO3 and deacetylates it. Deacetylation of FOXO3 induces activation of a subset of genes that promote resistance to oxidative stress (Brunet *et al.*, 2004). Among the FOXO targets activated by SIRT1 is SOD2, which has been shown to lead to reduction of oxidative stress in skeletal muscle cells (Pardo and Boriek, 2012). Another FOXO target that is activated by SIRT1 is catalase, which has been shown to be responsible for SIRT1-mediated protection of the heart from oxidative stress (Alcendor *et al.*, 2007). SIRT1 has also been shown to inhibit oxidative stress produced by the NF-kB pathway (Salminen *et al.*, 2008) and promote antioxidant targets of the transcription factor peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) (Pfluger *et al.*, 2008). Finally, a SIRT1 activator was shown to mitigate oxidative stress-induced loss of neurons in a mouse model of neurodegeneration (Khan *et al.*, 2014).

High Fat Diet

High fat diets (HFDs) result in various stresses on the body, including inflammation, which can ultimately result in insulin resistance and metabolic syndrome (Ogden *et al.*, 2007; Tschop and Thomas, 2006). As obesity is a major epidemic, especially in developed countries, there is a strong demand for interventions that can ameliorate some of its detrimental effects (Foster-Schubert and Cummings, 2006). As sirtuins are activated by calorie restriction and mediate some its benefits, they have been heavily investigated as agents that could protect against the detrimental effects of HFDs. SIRT1 transgenic mice placed on normal diets were found to have some phenotypes that resemble calorie-restricted mice, but did not have lifespan extension (Bordone *et al.*, 2007).

However, when SIRT1 transgenic were placed on HFD, they were found to be protected from numerous obesity-associated maladies, including hepatic steatosis, hepatic glucose intolerance, and hepatic inflammation (Pfluger *et al.*, 2008). Mechanistically, the protective effect of SIRT1 has been attributed to its inhibition of NFkB-induced inflammation and upregulation of the antioxidant targets of PGC-1α (Pfluger *et al.*, 2008). Recently, SIRT7 has also been shown to protect against HFD-induced hepatic inflammation and fatty liver (Shin *et al.*, 2013). SIRT7 exerts its protective effect by combating endoplasmic reticulum stress, which has been shown to contribute to a host of metabolic abnormalities induced by an HFD (Hotamisligil, 2010).

The Relation Between Stress and Aging

A common theme that has emerged from the pathways that improve health and/or increase lifespan is that they are responses to stresses that improve the cell's ability to cope with stress. In the case of CR, the stressor is a decrease in nutrients, which triggers a decrease in the activity of insulin/IGF-1 signaling and/or mTOR signaling. This leads to the initiation of a protective response that consists of various coping mechanisms such as increased transcription of stress resistance genes by FOXO, increased autophagy to recycle defective cellular components, and decreased translation to decrease the protein load of the cell (Kenyon, 2010). Depending on context, CR has also been shown to lead to an increase in both sirtuin expression (Palacios *et al.*, 2009, Shi *et al.*, 2005) and activity through increasing the NAD+/NADH ratio level in the cell (Lin *et al.*, 2004). As shown in the preceding section, sirtuins are able to combat various different types of stresses to either increase lifespan and/or improve cell and organismal function.

CR combats aging not through decreasing metabolism, but by activating a specific genetic program to increase stress resistance and improve cellular function. It is for this reason that the benefits of CR can be uncoupled from being placed on CR. By simply activating the genetic pathways that are triggered by CR, the health/lifespan benefits of CR can be realized. In the following section, two other stress responses and their relations to aging will be discussed.

Oxidative Stress and Aging

One of the oldest theories of aging is the free radical theory of aging, and it postulates that aging is caused by cellular damage inflicted by ROS (Harman, 1956). Support for this theory stems from the observation that levels of oxidative stress and damage generally increase with age (Sohal and Weindruch, 1996) and studies that have shown that deleting SOD, a major scavenger of ROS, results in reduced lifespans (Wawryn *et al.*, 1999; Kirby *et al*,. 2002). However, some major questions have arisen over the years with regards to how much of the connection between oxidative stress and aging is causative as opposed to

being correlative (Brown *et al.*, 2012). Is reduction of oxidative stress by itself sufficient to increase lifespan and/or prevent aging-associated diseases?

A direct way of addressing this question is to simply add exogenous antioxidants to the diet and determine whether they lead to any beneficial effects on lifespan or health. Numerous studies have been performed with antioxidant supplementation, and the results have been very discouraging. It appears that antioxidants in general have very little effect on lifespan or disease and in some cases can be deleterious (Ernst *et al.*, 2013, Bernhard and Wang, 2007, Magwere *et al.*, 2006, Bjelakovic *et al.*, 2004). Thus, simply removing ROS seems insufficient to confer major benefits. A possible solution to this dilemma is illustrated by the case of the naked mole rat (*Heterocephalus glaber*), which despite living extraordinarily long for a rodent (upward of 30 years), actually has similar levels of ROS production and paradoxically, may actually have higher biomarkers for oxidative damage (Andziak *et al.*, 2006). A key finding however, is that the naked mole rat has higher resistance to oxidative stress-induced apoptosis (Labinskyy *et al.*, 2006). Thus, analogous to the case of CR, it is not the reduction of ROS that mediates age-related benefits, it is the genetic program that responds to ROS.

Among the responders to ROS are antioxidant enzymes such as SOD and catalase. Studies overexpressing these enzymes have had mixed success in extending lifespan (Orr and Sohal, 1994, Perez *et al.*, 2009). However, this could be due to the difficulty in properly localizing these enzymes when overexpressed or properly activating their enzymatic activity. Targeting catalase specifically to mitochondria has resulted in lifespan extension in mice (Schriner *et al.*, 2005). Additionally, a study has shown that overexpressing SOD2 by itself only has a small effect on ROS, as it needs to be deacetylated by SIRT3 to have full enzymatic activity (Qiu *et al.*, 2010). It will be interesting to determine whether SIRT3 and SOD2 double transgenic mice will have extended lifespans.

The importance of the cellular response to ROS as opposed to the level of ROS itself in determining lifespan is demonstrated by studies that have shown that lifespan extension from modulating antioxidant enzymes can be uncoupled from the levels of ROS. Instead of simply lowering ROS levels, antioxidant enzymes may promote lifespan by triggering other stress-resistance pathways, which then promote overall cellular resistance to stress (Van Raamsdonk and Hekimi, 2009, Cabreiro *et al.*, 2011). Thus, in a similar scenario to CR, it is the downstream stress response program to oxidative stress that ultimately affects lifespan.

Proteostasis and Aging

Proteostasis, maintenance of protein homeostasis, is essential for proper organismal and cellular function and a breakdown in proteostasis has been associated with multiple age-related degenerative diseases (Powers *et al.*, 2009). Protein aggregation and misfolding place stresses on the cell's machinery for

degrading and properly folding proteins, which varies depending on the cellular compartment: cytosolic unfolded proteins are handled by the heat shock proteins (HSPs) and the heat shock response, unfolded proteins in the endoplasmic reticulum are handled by the unfolded protein response (UPR), and unfolded proteins in the mitochondria are handled by the mitochondrial UPR (mtUPR) (Kourtis and Tavernarakis, 2011; Haynes and Ron, 2010).

At the most essential level, proteostasis is a balance between production through translation and degradation via the ubiquitin-proteasome system (UPS). Interestingly, inhibition of translation has been shown to extend lifespan in numerous organisms (Kaeberlein *et al.*, 2005, Steffen *et al.*, 2008, Curran and Ruvkun, 2007, Pan *et al.*, 2007). This is possibly due to a reduction in protein load to allow more time for chaperones to properly fold proteins and/or a stress response that mimics CR and leads to similar gene expression changes (Kaeberlein and Kennedy, 2011). Indeed, in the case of ER stress, which results from accumulation of misfolded proteins in the ER (discussed more in chapter 2), inhibition of translation is essential for restoration of homeostasis (Hetz, 2012). Deficiency of ribosomal subunits, which impairs translation, has been shown to result in increased resistance to ER stress (Steffen *et al.*, 2012).

Although it would be logical to think that the cell would be healthiest without any stress from misfolded or aggregated proteins, absence of any stress does not lead to maximum lifespan. As in the case with CR and oxidative stress, a genetic stress resistance pathway must be activated to confer lifespan extension. In the case of both ER stress and mitochondrial stress, a mild level of stress leads to beneficial activation of the UPR and mtUPR pathway, which leads to lifespan extension (Salminen and Kaarniranta, 2010, Houtkouper *et al.*, 2013). However, chronic elevated stress leads to apoptosis (Salminen and Kaarniranta, 2010).

Concluding Themes

A major theme that emerges from this review of cellular responses to stress is that with regards to longevity, maybe Nietzsche was correct after all: "What does not kill me makes me stronger". Using more pertinent terminology, the concept of hormesis is echoed throughout the pathways that affect aging. Mild levels of stress are beneficial, resulting in upregulation of stress pathways that lead to protection and increased lifespan and health span. CR is in essence a nutritional stress that results in enhanced stress resistance. Mild oxidative stress in the mitochondria leads to mitohormesis, improving mitochondrial function and extending lifespan (Ristow and Zarse, 2010). Finally, mild protein folding stress, by activating the protective UPR response, also leads to increased longevity (Salminen and Kaarnirantha, 2010). With regards to longevity, a slightly bumpy ride takes you farther than a perfectly smooth one.

Another theme is that the various longevity pathways are intimately connected. CR leads to the activation of responses to oxidative stress (Qiu *et al.*,

2010), while inhibiting ribosomal subunits leads to activation of AMPK, which results in a metabolic pattern reminiscent of CR (Kaeberlein and Kennedy, 2011). Sirtuins, which were originally found to maintain genomic integrity, have now been linked to the response to CR and also oxidative stress. In the following two chapters, the web of interconnectedness of the various stress pathways will be woven even more intricately with a novel connection between sirtuins and proteostasis.

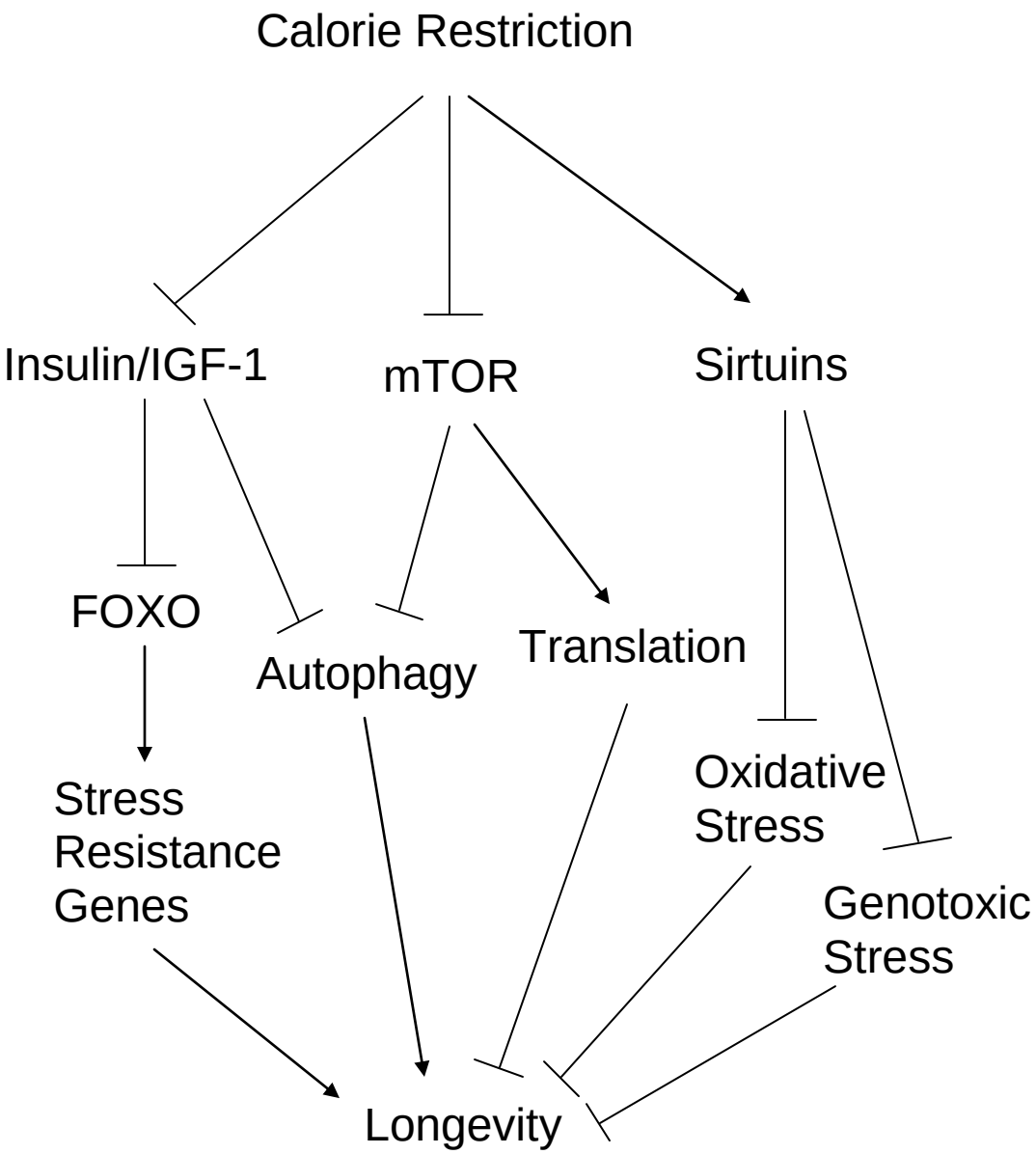


Fig. 1.1: Pathways regulating longevity
Calorie restriction regulates the activity of 3 different pathways that affect longevity. Insulin/IGF-1 signaling is inhibited, which allows FOXO to upregulate the expression of stress resistance genes that promote longevity. mTOR is also inhibited, which leads to a reduction in translation that has been shown to promote longevity. Inhibition of both mTOR and insulin/IGF-1 leads to induction of autophagy, which increases longevity. The expression and activity of sirtuins is increased. Sirtuins have been shown to combat genotoxic and oxidative stress, which have been shown to contribute to aging.

Chapter 2

SIRT7 and the Cellular Response to Endoplasmic Reticulum Stress

Introduction

Eukaryotic cells are like factories in miniature. They have division of labor in the form of various different organelles that perform diverse functions. To ensure harmony, as in any factory, supply and demand must be balanced. For cells, proteins are one of the major products that are produced, and the production process is subject to tight regulation. One of the major quality control facilities in the cell is the endoplasmic reticulum (ER), which ensures the proper folding of various secreted proteins through a complement of ER-associated chaperone proteins that aid in folding (Hetz, 2012).

Accumulation of misfolded proteins in the ER leads to the activation of a set of cellular responses known collectively as the unfolded protein response (UPR) that works to restore homeostasis. The logic of the UPR is to transduce information about misfolded proteins from the ER to the nucleus where a transcriptional response can be initiated that leads to corrective action back in the ER. The corrective actions include a decrease in the rate of translation and an increase in ER chaperones, among other responses. The UPR consists of three distinct complementary pathways that are initiated by the stress sensing proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (Fig. 2.12) (Walter and Ron 2011).

PERK is a transmembrane ER receptor that is activated via dimerization and autophosphorylation. Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which then results in suppression of general translation (Walter and Ron 2011). In addition, eIF2α promotes the translation of activating transcription factor 4 (ATF4), which has been shown to promote the transcription of genes involved in autophagy and protein folding (B'chir *et al.*, 2013).

IRE1α, like PERK, is a transmembrane ER receptor and is activated under ER stress conditions via dimerization and autophosphorylation. The activation of IRE1α enables an RNase activity, that allows it to activate the transcription factor X box-binding protein 1 (XBP1), by splicing an intron in the mRNA encoding the inactive version of the protein (XBP1u), converting it to the active spliced XBP1s. XBP1s translocates to the nucleus where it activates chaperones and genes involved in ER-associated degradation (ERAD). IRE1α also decreases the levels of proteins entering the ER through degrading mRNAs encoding ER localized proteins in a process known as regulated IRE1-dependent decay (RIDD) (Walter and Ron 2011).

Lastly, ATF6 is normally localized to the ER membrane, but translocates to the Golgi via COPII vesicles under ER stress conditions, where it is cleaved by

site 1 protease (S1P) and site 2 protease (S2P). Cleavage of ATF6 generates an active transcription factor, ATF6f, that translocates to the nucleus and activates XBP1 and genes associated with ERAD (Walter and Ron 2011).

The resolution of ER stress is of vital importance to the cell as chronic stress can lead to apoptosis through various pathways. One of the most well-known apoptotic pathways is via C/EBP-homologous protein (CHOP), which is regulated transcriptionally by the PERK downstream target ATF4. CHOP itself is a transcription factor that promotes the transcription of the pro-apoptotic BCL-2 homology 3 (BH3)-only protein BCL-2-interacting mediator of cell death (BIM) and suppressing the transcription of the pro-survival protein B cell lymphoma 2 (BCL-2) (Sano and Reed, 2013). ATF4 and p53 have also been shown to lead to the activation of ER pro-apoptotic BH3-only proteins under ER stress conditions (Sano and Reed, 2013).

The importance of ER stress management extends well beyond the level of the cell. Chronic ER stress has been implicated in inflammation and metabolic dysfunction. Non-alcoholic fatty liver disease (NAFLD), insulin resistance, diabetes, and adipose tissue inflammation are among the pathologies that have been linked to chronic ER stress (Hotamisligil, 2010; Ozcan *et al.* 2004). As conserved regulators of lifespan and metabolism, sirtuins are prime candidates for responding to ER stress. However, very little work has been done to determine how sirtuins can regulate the ER stress response. In Drosophila, one study has shown that inhibiting the sirtuin homolog *sir2.1* can increase expression of genes involved in the UPR and extend lifespan (Viswanathan *et al.*, 2005). Meanwhile, another group has shown that the mammalian sirtuin SIRT1 suppresses the transcriptional activity of XBP1s and sensitizes cells to ER stress-induced cell death (Wang *et al.*, 2011).

A recent study on SIRT7 has shown that it possesses H3K18 deacetylase activity and is enriched at the promoters of genes enriched in metabolism and translation (Barber *et al.*, 2012). As acetylated H3K18 is associated with active transcription, deacetylation of H3K18 by SIRT7 leads to silencing of gene expression (Barber *et al.*, 2012). Given the important link between translation and the UPR, we investigated whether SIRT7 might play a role in the cellular response to ER stress, a connection which promises to not only shed light on the biology of SIRT7, one of the least studied mammalian sirtuins, but also possibly pave the way to novel approaches to tackle ER stress-related metabolic diseases.

Results

SIRT7 is Transcriptionally Upregulated by ER Stress

To determine whether SIRT7 might play a role in the UPR, we looked at the expression of SIRT7 under conditions of ER stress induction. Thapsigargin (TG) and tunicamycin (TM) are commonly used pharmacological ER stress inducers. The mechanism of action of TG is thought to occur through inhibition of

Ca^{2+} ATPases in the ER (Lytton *et al.*, 1991), while TM inhibits N-linked protein glycosylation (Kuo and Lampen 1974). Both processes are crucial to the ability of the ER to properly fold proteins.

Treatment of mouse embryonic fibroblasts (MEFs) and two liver cell lines, Hepa1-6 and HepG2, with TM led to a significant two-fold induction of SIRT7 mRNA in all three cell types (Fig. 2.1A). At the protein level, we also saw a significant induction of SIRT7 under both TM and TG treatment (Fig. 2.1B and 2.1C).

Having found induction of SIRT7 under conditions of ER Stress, we investigated which of the three pathways in the ER stress response regulates the transcription of SIRT7. A bioinformatic analysis of the SIRT7 promoter found multiple XBP1s consensus binding sites (Acosta-Alvear *et al.*, 2007). To determine whether SIRT7 is a target of XBP1s under ER stress conditions, we treated MEF cells generated from XBP1 knockout (KO) mice with TG and found that SIRT7 mRNA expression was not induced in these cells, while there was a robust induction in MEF cells generated from matched WT mice (Fig. 2.2A). Western blotting confirmed the findings at the protein level (Fig. 2.2B). Using a plasmid encoding XBP1s, we found that transfection of Hepa1-6 cells led to induction of SIRT7 to a similar extent as the canonical XBP1 target Erdj4 (Lee *et al.*, 2003) (Fig. 2.2C). We also confirmed this result at the protein level through Western blotting.

Deficiency of SIRT7 Results in Constitutive ER Stress

Having determined that SIRT7 is induced by ER stress, we explored whether deficiency of SIRT7 leads to a defect in the ER stress response. We infected 293T cells and HepG2 cells with lentiviral plasmids encoding two separate SIRT7 shRNA constructs (KD1 and KD2). Knockdown of SIRT7 led to an increase in ER stress in both cells lines, as evidenced by increased phosphorylation of eIF2α in both KD1 and KD2 (Fig. 2.3A and Fig. 2.3B).

We also isolated MEF cells from SIRT7 KO and littermate WT embryos. Upon quantitative real-time PCR analysis of WT and KO MEF cells, we found that numerous ER stress genes were elevated in the KO MEF cells (Fig. 2.4A). As an independent assay of ER stress, we collaborated with Patrick Li in the Kapahi Lab at the Buck Institute for Aging and obtained polysome profiles for the WT and KO MEF cells. In brief, polysome profiles show the ratio of polysomes to monosomes and free ribosomes (Masek *et al.*, 2011). Cells with large amounts of translation show a high polysome to monosome ratio, while cells undergoing ER stress, which leads to a reduction in translation, show a lower polysome to monosome ratio (Fu *et al.*, 2012). The polysome profile for KO MEF cells shows a markedly lower polysome to monosome ratio (Fig. 2.4B), which in conjunction with the gene expression data suggests that the cells may have elevated ER stress.

SIRT7 Protects Against ER Stress

To determine whether SIRT7 can protect against ER stress, we generated cells that had enforced expression of SIRT7 using a retroviral plasmid encoding SIRT7 (T7). When treated with TM, the cells overexpressing SIRT7 had reduced levels of ER stress as evidenced by reduced phosphorylation of eIF2α (Fig. 2.5A) and expression of ER stress genes (Fig. 2.5B) compared to control cells. Importantly, we showed that the reduction in ER stress by SIRT7 depends on its catalytic activity, as cells overexpressing a catalytically inactive SIRT7 mutant (HY -> substitution of Y for a conserved H at the 187th amino acid of SIRT7 in the conserved catalytic domain) did not have reduced ER stress (Fig. 2.5A and Fig. 2.5B).

Since chronic ER stress leads to apoptosis, we assayed cell survival in cells deficient in SIRT7 and cells that constitutively overexpress SIRT7. Both KD1 and KD2 cells had markedly lower cell survival upon TM treatment compared to control cells (Fig. 2.6A). Conversely, cells that overexpressed SIRT7 were more resistant to cell death induced by TM compared to control cells (Fig. 2.6B). These results suggest that SIRT7 is able to protect against ER stress and prevent chronic ER stress-induced cell death.

SIRT7 Suppresses ER Stress Through a Myc-Dependent Mechanism

The next question we addressed was how SIRT7 can play a role in the ER stress response. A previous study had found SIRT7 to function at chromatin as an H3K18 deacetylase, silencing gene expression (Barber *et al.*, 2012). SIRT7 was found to bind to the promoters of multiple ribosomal subunits, which indicates that it could function to suppress translation, which is a component of the cellular response to ER stress. However, SIRT7 does not have a DNA binding domain and must be directed to chromatin by a transcription factor. Since Myc is known to bind and regulate the transcription of ribosomal subunits (Riggelen *et al.*, 2010), we hypothesized that SIRT7 is directed to ribosomal subunits by Myc. We transfected Hepa1-6 cells with a Flag-tagged SIRT7 construct and performed immunoprecipitation with a Flag antibody, pulling down Myc (Fig. 2.7). Our lab has also found that Myc can be pulled down endogenously in cell lines using SIRT7 polyclonal antibody.

Myc can function to enhance or suppress transcription, depending on its cofactors (Eilers and Eisenman, 2008). Our hypothesis is that Myc promotes the transcription of ribosomal subunits, which promotes translation. Under ER stress conditions, translation must be inhibited, and we hypothesized that this may be partially due to upregulation of SIRT7, which then binds to Myc and leads to suppression of the transcription of Myc-targeted ribosomal subunits. In this way, less ribosomal subunits are produced and translation is inhibited. In the absence of SIRT7, Myc would only be able to positively regulate the transcription of ribosomal subunits, which will compromise the ability of the UPR to restore homeostasis, resulting in constitutive ER stress.

Having shown the interaction between Myc and SIRT7, we next determined whether inhibiting Myc could rescue the increased ER stress seen in SIRT7 KD cell lines. At both the mRNA and protein levels, we found that SIRT7 target ribosomal subunits (RPS14 and RPS20) were elevated in SIRT KD cell lines (Fig. 2.8A and Fig. 2.8C). The increase in the ribosomal subunits was abrogated when Myc was inhibited with a synthetic Myc inhibitor 10058-F4 (Huang *et al.*, 2006) (Fig. 2.8A and Fig. 2.8C). Importantly, Myc inhibition in SIRT7 KD cells did not rescue the elevation of NME1, a non-ribosomal target of SIRT7, suggesting that Myc specifically directs SIRT7 to ribosomal targets (Fig. 2.9B). This specificity was further confirmed by chromatin immunoprecipitation (ChIP) experiments done in our lab showing that the enrichment of SIRT7 to ribosomal subunit promoters, but not other SIRT7 targets, is significantly reduced when Myc is knocked down using siRNAs.

To investigate whether Myc is responsible for the increased ER stress in SIRT7 KD cells, we knocked down Myc in SIRT7 KD and control cell lines in the presence or absence of TM treatment. With or without TM treatment, SIRT7 KD cells had elevated levels of ribosomal subunits (RPS14 and RPS20) (Fig. 2.9). Analogous to the results with the Myc inhibitor (Fig. 2.8A and Fig. 2.8C), elevation of the ribosomal subunits is abrogated upon knockdown of Myc (Fig. 2.9). Also similar to previous results (Fig. 2.3), KD of SIRT7 led to a constitutive elevation of ER stress as shown by increased phosphorylation of eIF2α and increased expression of grp78 (Fig. 2.9). Treatment of the cells with TM led to a significant induction in ER stress, which was greater in the SIRT7 KD cells (Fig. 2.9). Knockdown of Myc rescued the constitutive ER stress in SIRT7 KD cells and also reduced the level of ER stress in TM-treated SIRT7 KD cells to the same level as the TM-treated control cells (Fig. 2.9). Thus, by abrogating the elevation of ribosomal subunits, knockdown of Myc rescued the elevated ER stress in SIRT7 KD cells.

Lastly, we tested whether Myc inhibition or knockdown could rescue the decreased survival of SIRT7 KD cells upon treatment with ER stress inducers. Using HepG2 cells, we found that SIRT7 KD cells had decreased cell survival compared to control cells when treated with TM. However, upon inhibition or knockdown of Myc, the survival of SIRT7 KD cells was rescued to the level of control cells.

Discussion

A New Component in the ER Stress Response

Our results add a new component to the cellular response to ER. Whereas traditionally the UPR has been thought to mediate inhibition of translation through phosphorylation of eIF2α via the PERK branch (Walter and Ron 2011), our work has shown that translation can also be inhibited at the transcriptional level through suppression of ribosomal subunits by SIRT7, downstream of XBP1s in the IRE1α branch. Our work has also connected the UPR to Myc activity through

SIRT7, which acts as a brake on Myc controlled transcription of ribosomal proteins under ER stress conditions.

Chronic ER stress has been shown to lead to apoptosis, and one mechanism that has been proposed is through p53, but how p53 is activated by ER stress is not currently known (Sano and Reed, 2013). However, it is known that persistent Myc activation can lead to activation of p53 (Felsher *et al.*, 2000). With the connection between Myc and ER stress shown in our study, it is possible that p53-mediated cell death under conditions of chronic ER stress is due to persistent increased Myc activity, possibly due to deficiency in SIRT7 expression or activity.

Implications for Fatty Liver Disease

Previous studies have shown a strong link between chronic ER stress and metabolic disorders such as fatty liver (Hotamisligil, 2010; Ozcan *et al.* 2004). With the mechanistic findings of this study, it is tempting to speculate on whether SIRT7 can used to treat conditions arising from chronic ER stress. Indeed, our lab has characterized a SIRT7 KO mouse model that develops fatty liver with essentially 100% penetrance (Shin *et al.*, 2013). In collaboration with Patrick Li at the Buck Institute, we performed polysome profiling on WT and SIRT7 KO livers and have shown that like the SIRT7 KO MEF cells (Fig. 2.4B), SIRT7 KO livers have a significant decrease in the ratio of polysomes to monosomes and free ribosomes (Fig. 2.11). In conjunction with other studies performed in our lab, we have strong evidence that constitutive ER stress in the livers of SIRT7 KO mice is responsible for the fatty liver disease. Our lab has shown that knocking down SIRT7 or Myc specifically in the livers of KO mice using an adeno-associated virus 8 (AAV8) vector leads to rescue of the fatty liver phenotype (Shin *et al.*, 2013). Interestingly, overexpressing SIRT7 using the AAV8 vector in the livers of WT mice protects against high-fat diet induced fatty liver disease (Shin *et al.*, 2013). Thus, we have already shown that the mechanistic link between SIRT7 and ER stress can be used as a tool to target metabolic disease.

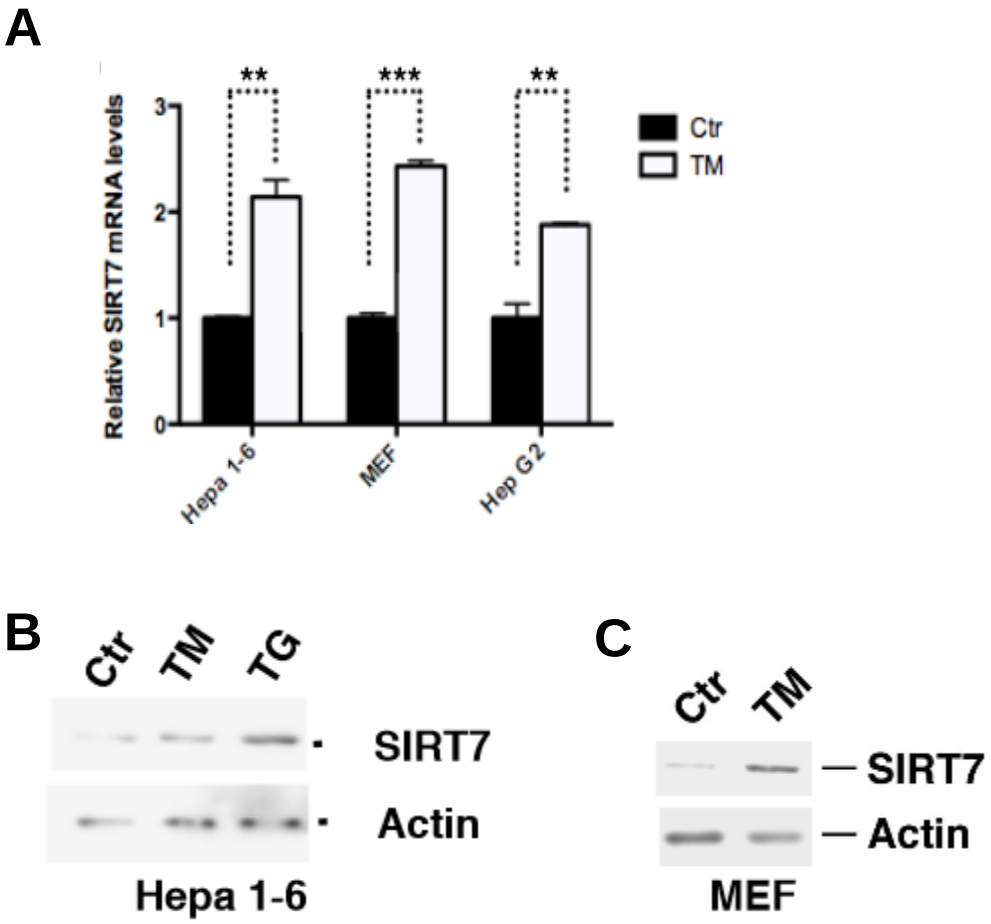


Fig. 2.1: SIRT7 is induced by treatment with ER stress inducers.
(A) Quantitative RT-PCR showing increase in SIRT7 expression upon treatment of Hepa1-6, MEF, and HepG2 cells with the ER stress inducer tunicamycin (TM).
(B) Western blot showing increase in SIRT7 protein levels in Hepa1-6 cells after treatment with the ER stress inducers TM or thapsigargin (TG).
(C) Western blot showing increase in SIRT7 protein levels in MEF cells after treatment with TM.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

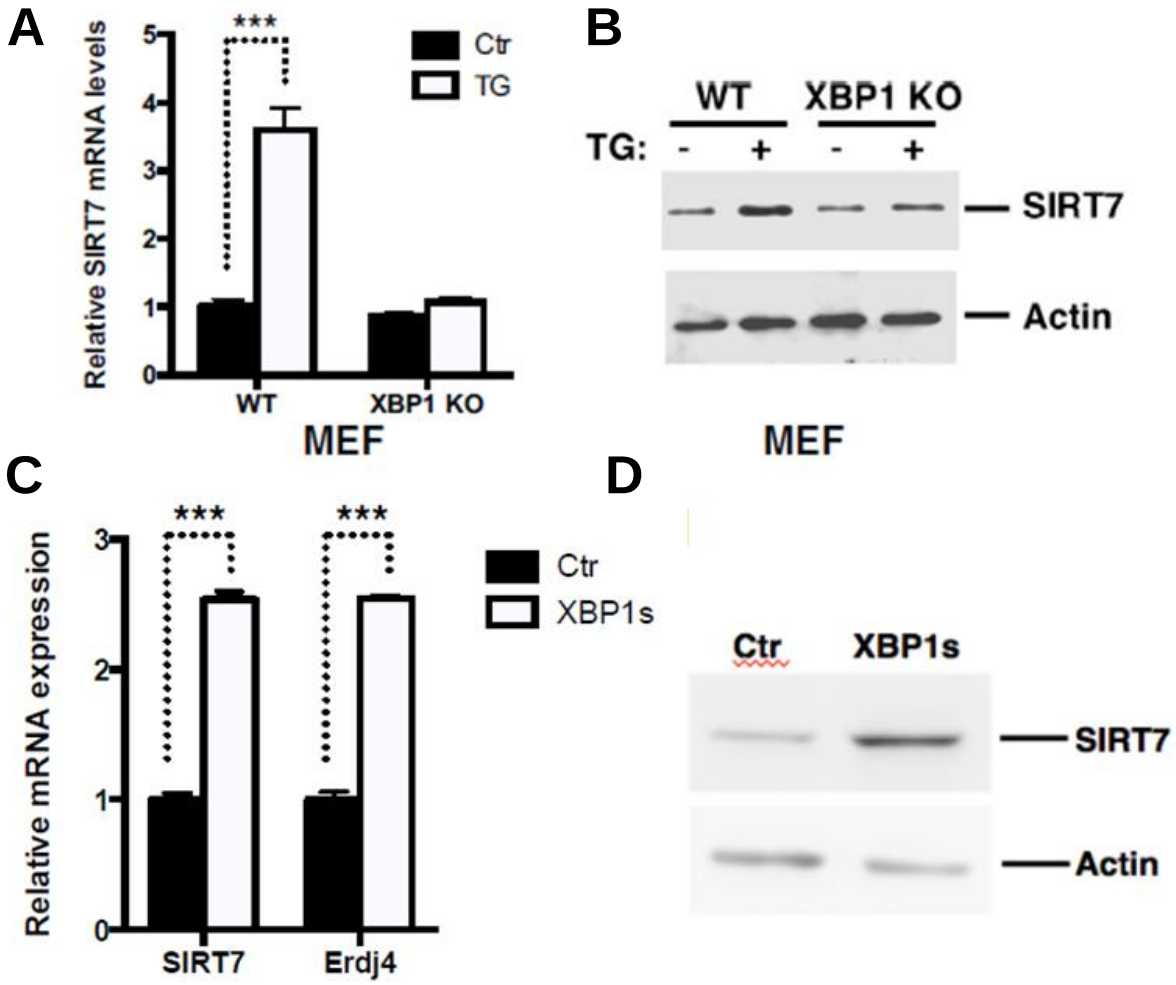


Fig. 2.2: SIRT7 induction upon ER Stress is dependent on XBP1
(A-B) Quantitative PCR (A) and western blot (B) showing increase in SIRT7 expression in WT MEF cells upon TG treatment and absence of increase in SIRT7 expression upon TG treatment in XBP1 KO MEF cells.
(C) Quantitative PCR showing that XBP1s leads to induction of SIRT7 to similar levels as Erdj4, a known target.
(D) Western blot showing that XBP1s leads to induction of SIRT7.
Error bars represent SEM: *: p<0.05, **: p<0.01, ***: p<0.001, ns: p>0.05

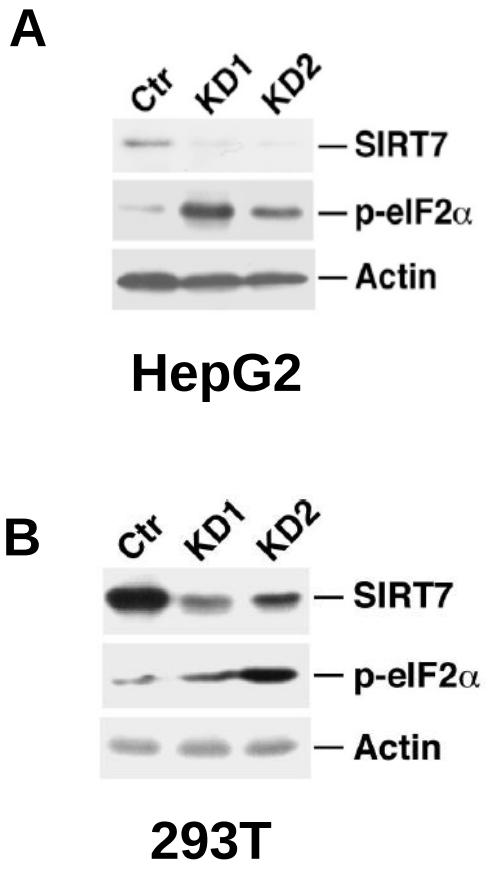


Fig. 2.3: Knockdown of SIRT7 leads to increased expression of ER Stress markers

(A-B) Western blots showing that two independent SIRT7 knockdowns made in HepG2 (A) and 293T cells (B) have increased phosphorylation of eIF2α, a marker of ER stress.

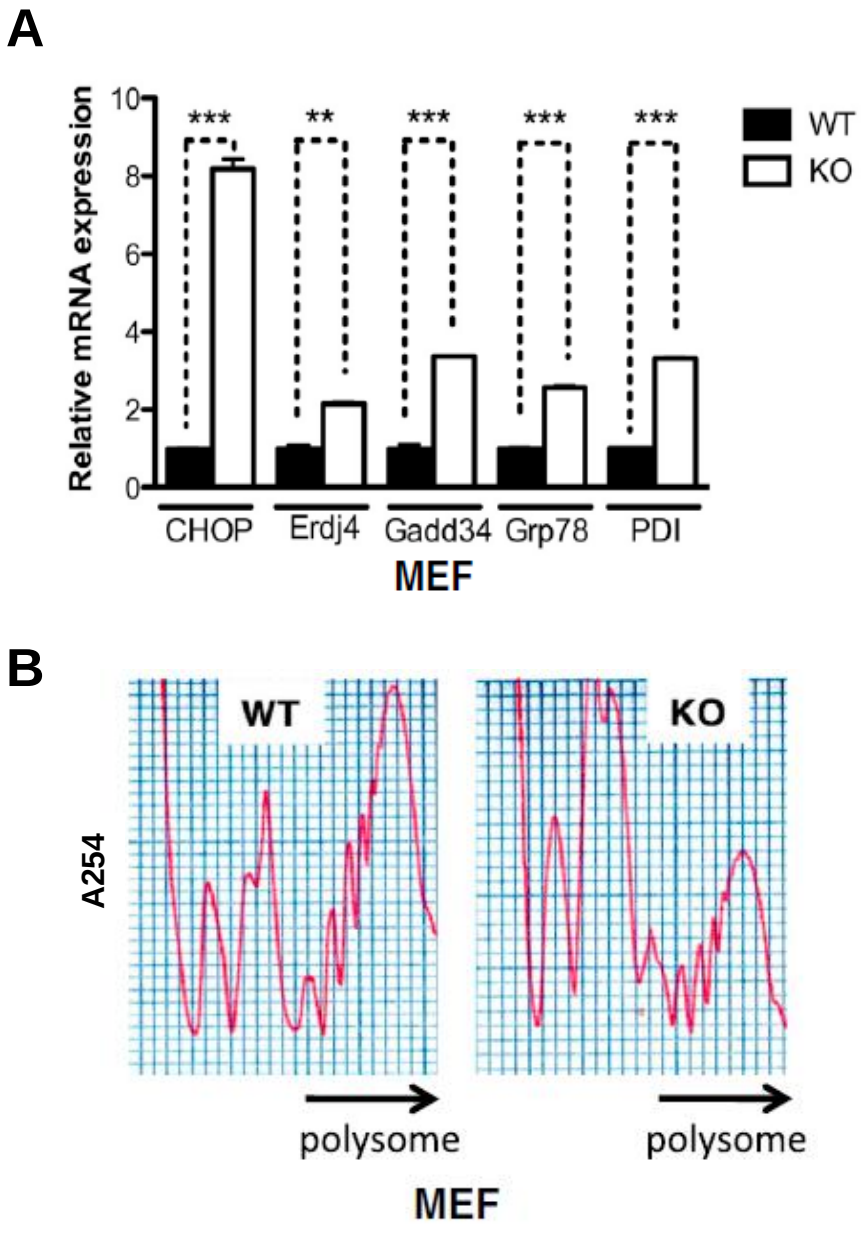
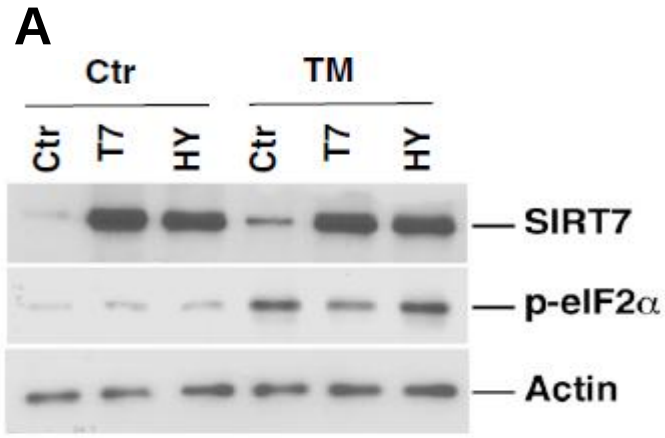


Fig. 2.4: SIRT7 KO MEF cells have elevated ER stress
(A) Quantitative PCR showing increased expression of ER stress genes in SIRT7 KO MEF cells compared to WT MEF cells.
(B) Polysome profile showing decrease in the ratio of polysomes (rightmost peak) to monosomes and free ribosomes (leftmost two peaks) in SIRT7 KO MEF cells compared to WT MEF cells.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$



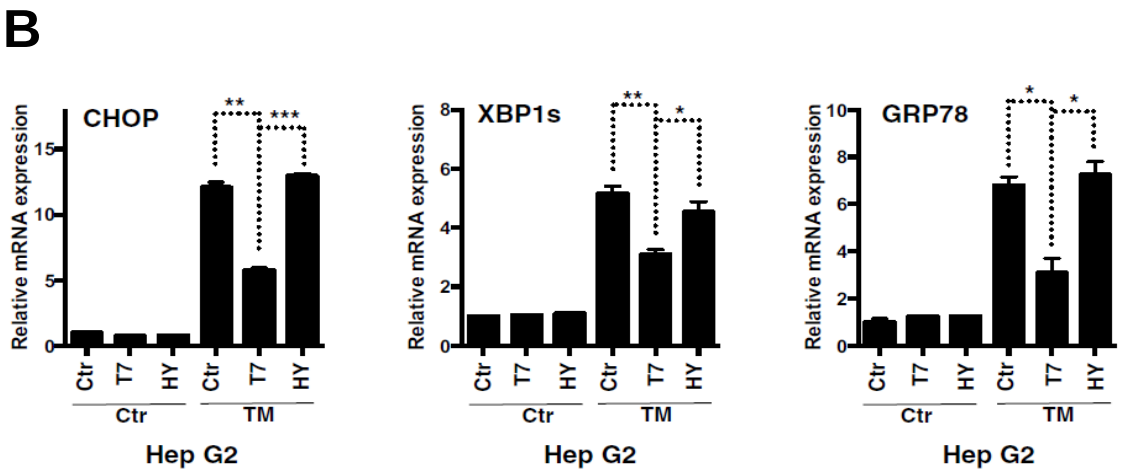


Fig. 2.5: SIRT7 protects against ER stress induction
(A) Western blot showing decreased phosphorylation of eIF2α in TM-treated cells overexpression SIRT7 (T7) compared to control (Ctr) and cells overexpressing a catalytically inactive SIRT7 (HY).
(B) Quantitative PCR showing decreased expression of the ER stress genes CHOP, XBP1s, and GRP78 in TM-treated cells overexpression SIRT7 (T7) compared to control cells (Ctr) and cells overexpressing a catalytically inactive SIRT7 (HY).
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

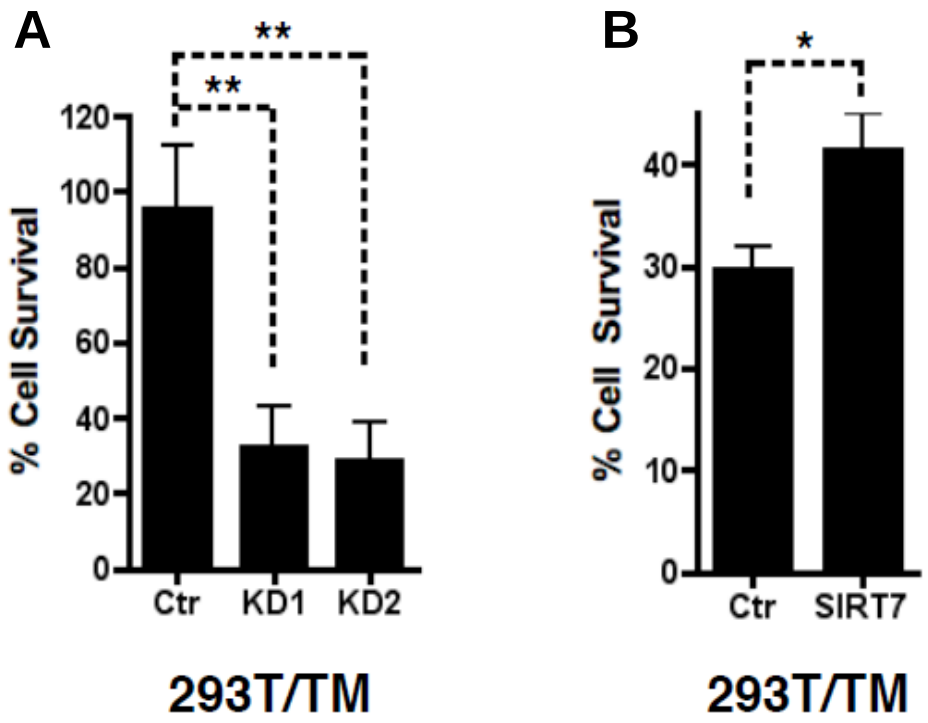


Fig. 2.6: SIRT7 protects against ER stress-induced cell death
(A-B) 293T cells with SIRT7 knocked down have less cell survival than control cells when treated with 1ug/mL of TM for 48 hours (A). 293T cells overexpressing SIRT7 have increased cell survival than control cells when treated with 2ug/mL of TM for 48 hours (B). Cell survival was determined with Trypan blue stain.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

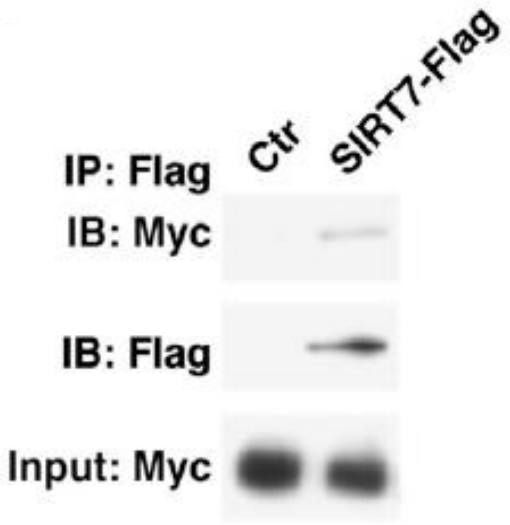


Fig. 2.7: SIRT7 Binds to Myc
Western blot showing co-immunoprecipitation of Flag-tagged SIRT7 and endogenous Myc in 293T cells.

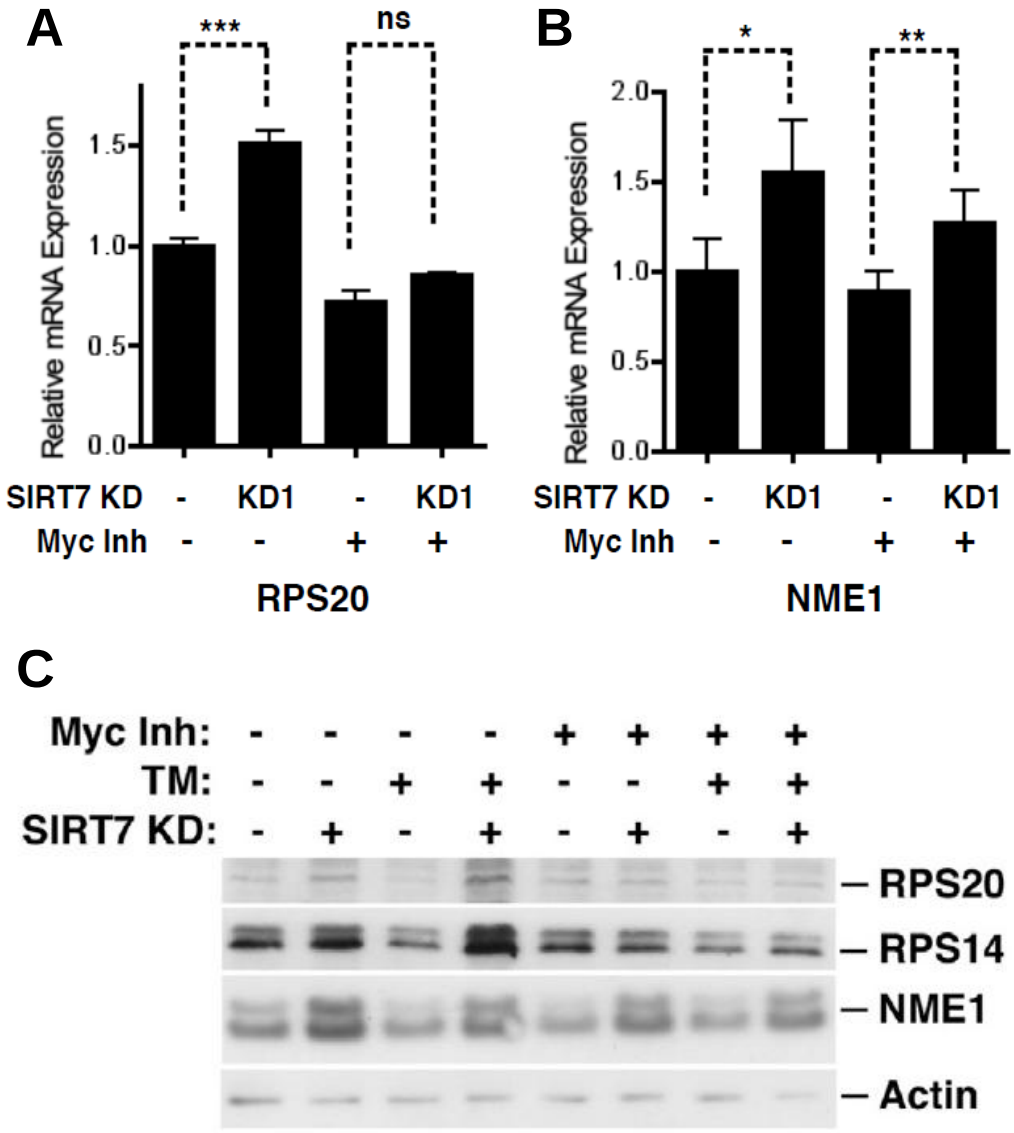


Fig. 2.8: Inhibition of Myc rescues the increased expression of SIRT7 ribosomal targets, but not other SIRT7 targets.
(A-B) Quantitative PCR showing that inhibiting Myc decreases the expression of RPS20 (A), but not NME1 (B), in a SIRT7 KD cell line.
(C) Western blot showing that inhibiting Myc decreases the expression of RPS14 and RPS20, but not NME1, in a SIRT7 KD cell line.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

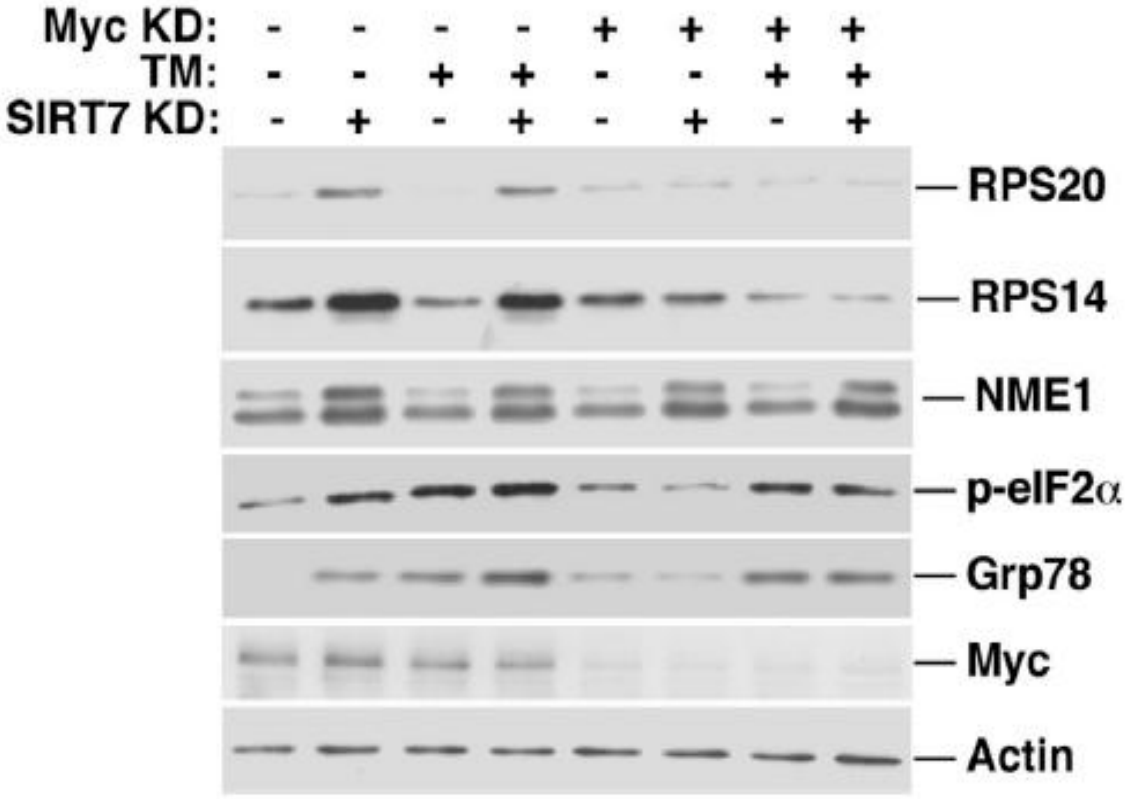


Fig. 2.9: Knockdown of Myc rescues the increased expression of SIRT7 ribosomal targets and increased ER stress.
Western blot showing that knocking down Myc via siRNA abrogates the increased expression of ribosomal subunits (RPS14 and RPS20) and ER stress markers (p-eIF2α and Grp78) in SIRT7 KD cells.

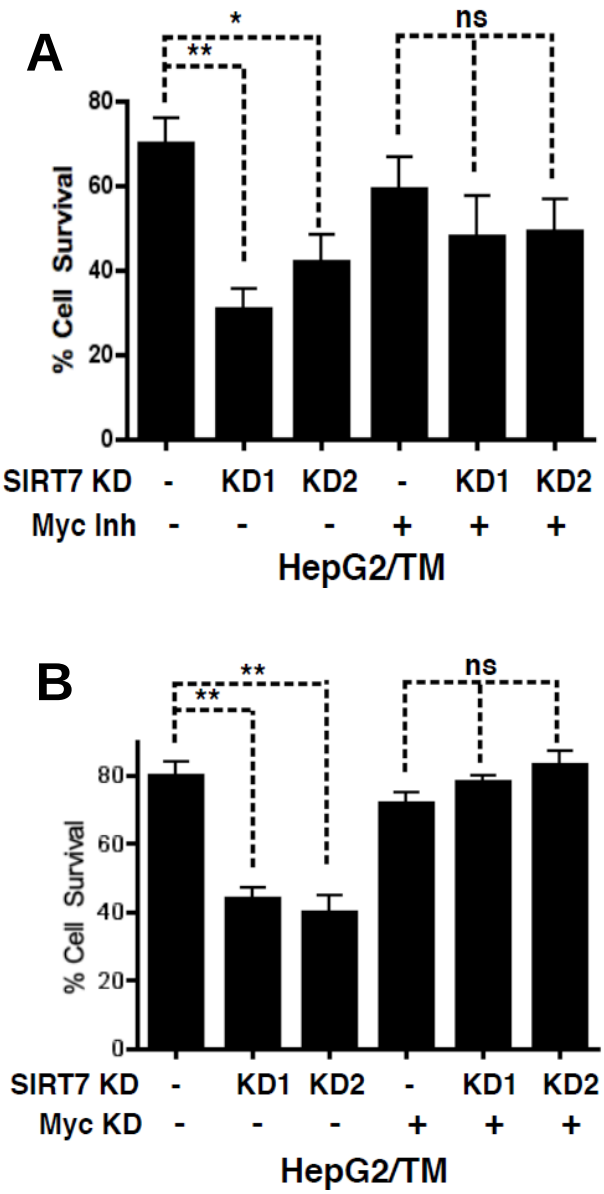


Fig. 2.10: Inhibition or knockdown of Myc rescues the decreased survival of SIRT7 deficient cells upon ER stress induction.
(A-B) Application of Myc inhibitor (A) or knockdown of Myc (B) rescues the decreased cell survival of SIRT7 KD HepG2 cells upon treatment with the ER stress inducer TM.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

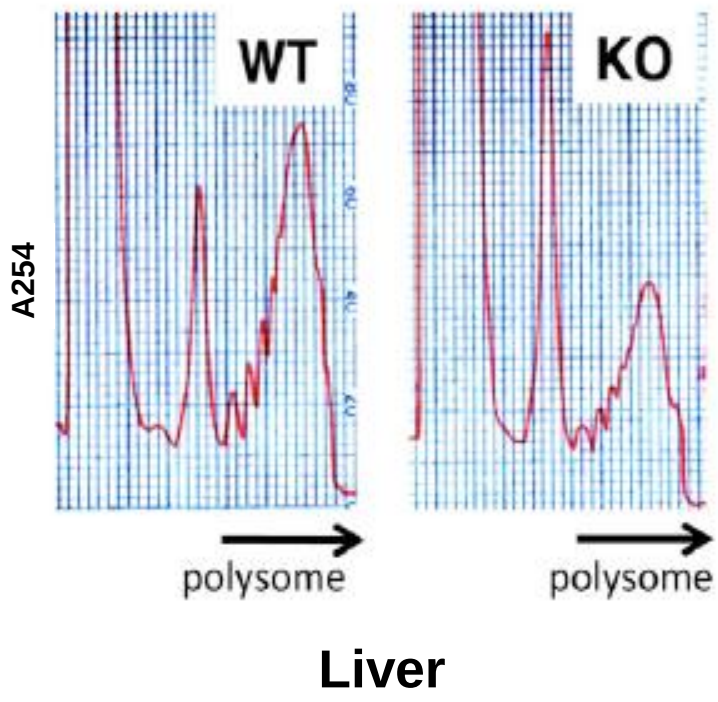


Fig. 2.11: SIRT7 KO livers have a reduced polysome to monosome ratio. Polysome profile showing decrease in the ratio of polysomes (rightmost peak) to monosomes and free ribosomes (leftmost two peaks) in SIRT7 KO liver compared to WT liver.

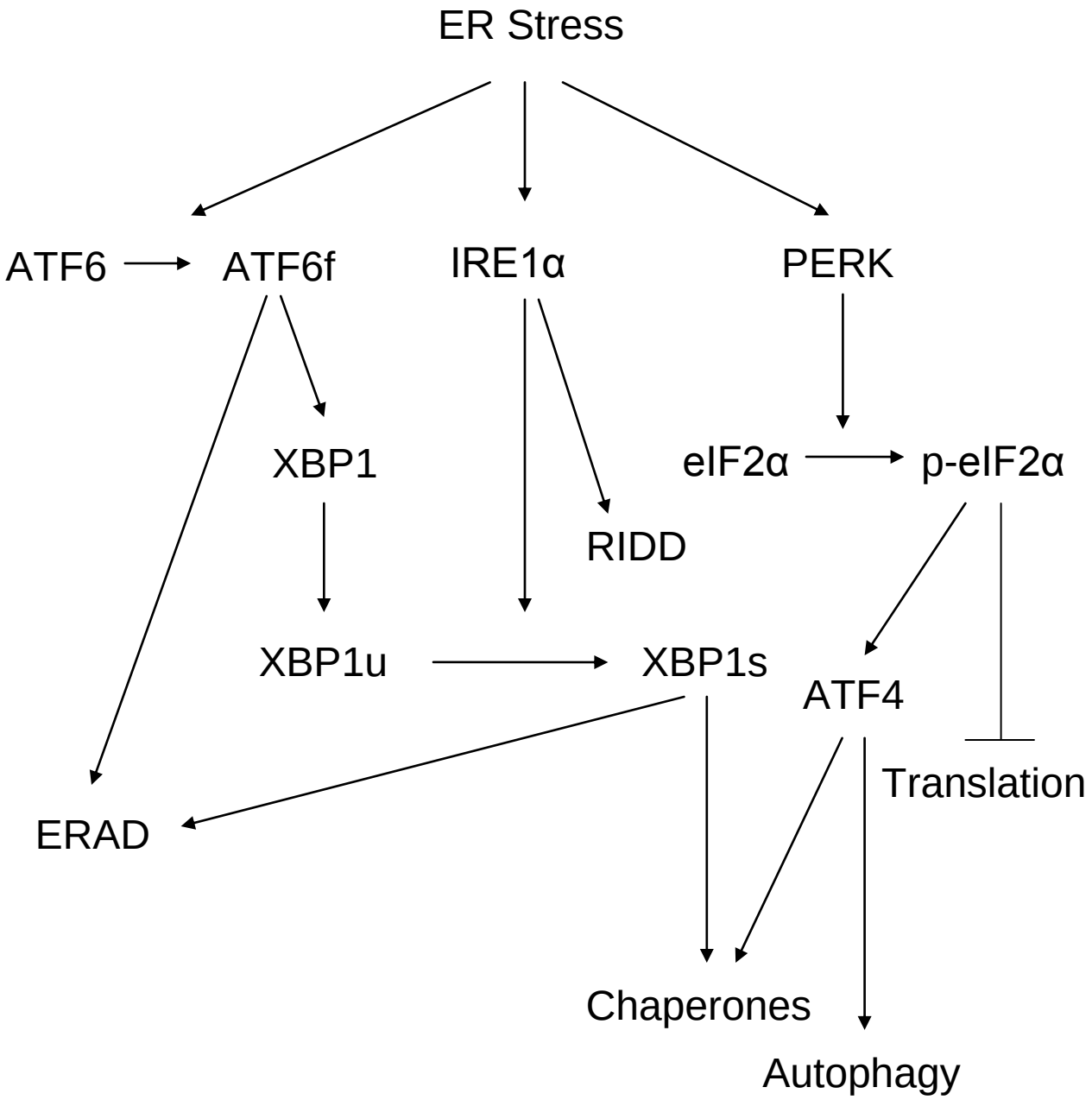


Fig. 2.12: Schematic of the three main ER stress response pathways. ER stress activates ATF6, IRE1α, and PERK to suppress translation, increase the expression of chaperones, and promote autophagy.

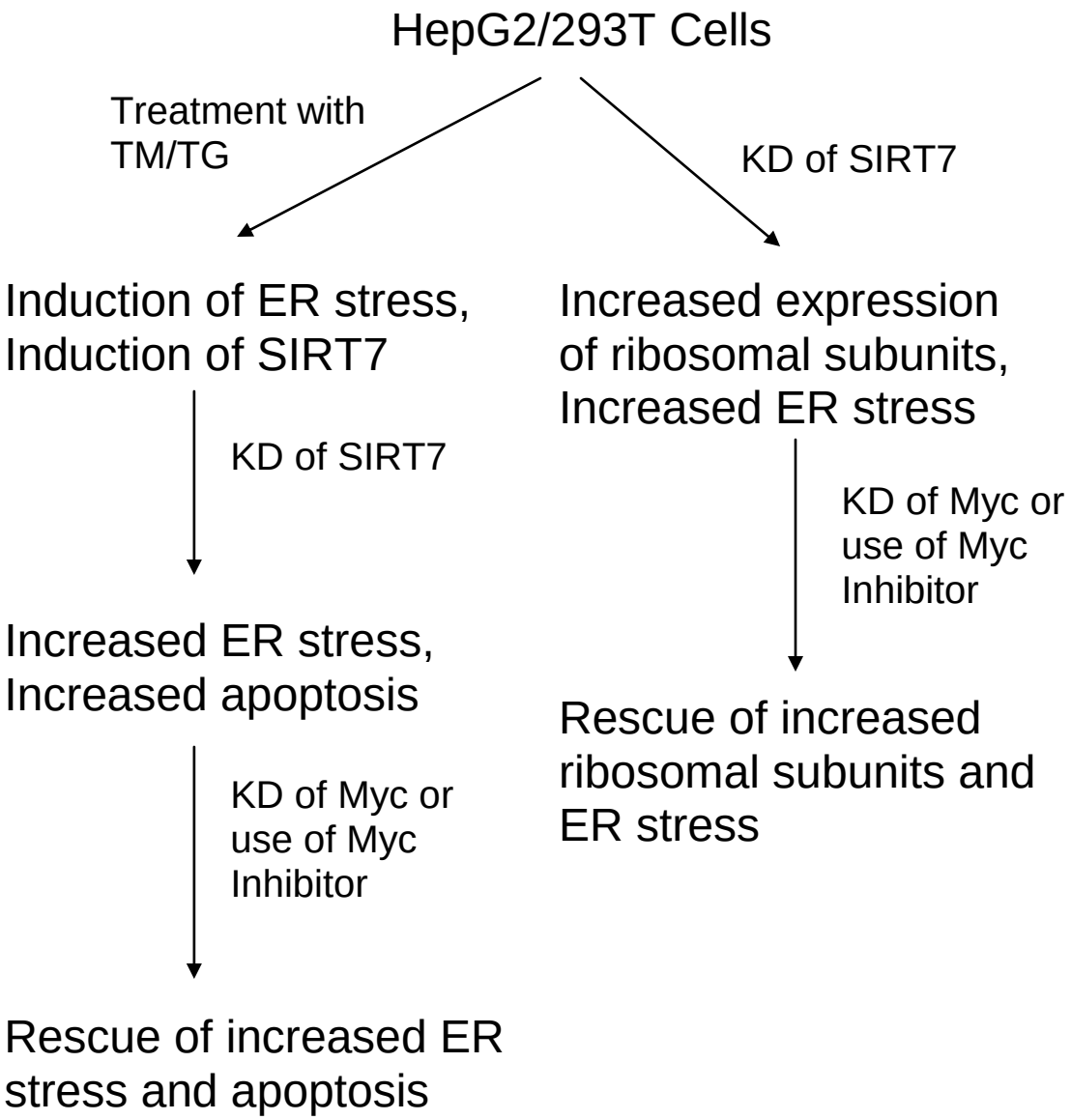


Fig. 2.13: Schematic of experimental design.
HepG2 cells were treated with TM or TG to induce ER stress. The stress was increased with SIRT7 KD, which led to increased apoptosis. KD or inhibition of Myc rescued the increased ER stress and apoptosis. KD of SIRT7 by itself induces an increase in ribosomal subunits and ER stress, which is also rescued by Myc inhibition or KD.

Chapter 3

SIRT7 and the Mitochondrial Unfolded Protein Response

Introduction

The ER is not the only location in the cell that is subject to protein trafficking and misfolding-related stress. Mitochondria contain their own genomes in the form of mtDNA, which encodes 13 genes (Boore, 1999). Analogous to ribosomes in the cytoplasm, mitochondria also have their own complement of mitochondrial ribosomes that perform translation of mitochondrial-encoded genes. In addition, mitochondria have numerous chaperones that aid in the folding of both mitochondrial-encoded proteins and nuclear-encoded proteins that are imported to the mitochondria. Accumulation of unfolded proteins in the mitochondria results in mitochondrial stress, which activates the mitochondrial unfolded protein response (mtUPR), leading to transcription of a set of nuclear genes that localize to the mitochondria and restore homeostasis (Haynes and Ron, 2010).

The details of the mtUPR are relatively poorly studied relative to ER stress. It is thought that mitochondrial stress is triggered by disturbances in mitochondrial homeostasis, such as bursts of mitochondrial biogenesis (Pellegrino *et al.*, 2013) or deviations in the stoichiometric ratios of nuclear and mitochondrial-encoded proteins, which can be induced by pharmaceutical interventions (Houtkouper *et al.* 2013).

The end result of chronic unresolved mitochondrial stress is thought to be either mitophagy, where the stressed mitochondrion is eaten up by the cell, or in extreme cases where the mitochondrial membrane potential becomes disturbed, apoptosis (Haynes and Ron, 2010). However, the details of the latter mechanism are still poorly understood.

Previous work in our lab on the role of SIRT7 in ER stress (Shin *et al.* 2013) led us to consider whether SIRT7 may play a role in mitochondrial stress as well. ChIP-seq analysis has shown that not only does SIRT7 bind to the promoters of ribosomal subunits, it also binds to the promoters of numerous mitochondrial ribosomal subunits and mitochondrial translation proteins (Barber *et al.*, 2012). Additionally, an immunoprecipitation and mass spectrometry study previously performed in our lab using SIRT7-Flag transfected 293T cells revealed NRF-1 as one of the proteins that was pulled down by SIRT7-Flag. NRF-1 is a transcription factor that serves as a master regulator of mitochondrial biogenesis and is bound to numerous mitochondrial genes (Kelly and Scarpulla, 2004). Thus, we wondered whether SIRT7 could be directed by NRF-1 to genes regulating mitochondrial translation, analogous to how SIRT7 is directed by Myc to ribosomal subunits. If so, a new link may potentially be forged between SIRT7 and the regulation of mtUPR.

Results

SIRT7 is Directed by NRF-1 to the Promoters of Mitochondrial Translation Genes and Mitochondrial Ribosomal Proteins

To confirm the results of the mass spectrometry analysis, we performed a Flag immunoprecipitation in 293T cells overexpressing SIRT7-Flag, which pulled down NRF-1 (Fig. 3.1A). We were also able to pull down NRF-1 endogenously using SIRT7 polyclonal antibody in 293T cells (Fig. 3.1B). Having established that SIRT7 interacts with NRF-1 either directly or in a complex, we next assayed whether NRF-1 binds to the promoters of SIRT7 mitochondrial targets at the same location that SIRT7 binds. Referencing a previously published SIRT7 ChIP-seq dataset (Barber *et al.*, 2012), our lab designed primers within the regions that were bound by SIRT7. Performing ChIP with NRF-1 polyclonal antibody, we found strong enrichment of NRF-1 at the promoters of SIRT7 mitochondrial targets (GFM2 and MRPL24) (Fig. 3.2A). Importantly, we found that the binding of NRF1 is specific to the mitochondrial targets of SIRT7 and not control targets (Tubulin) or non-mitochondrial targets of SIRT7 (RPS20 (Shin *et al.* 2013) and NME1 (Barber *et al.* 2012)) (Fig. 3.2B).

To establish that SIRT7 is directed to mitochondrial promoters by NRF1, our lab performed knockdown of NRF1, which dramatically reduced the enrichment of SIRT7 at the promoters. We found that NRF1 specifically mediates the binding of SIRT7 to mitochondrial promoters as knockdown did not decrease enrichment at other SIRT7 targets.

SIRT7 KD Cells have NRF-1-dependent Elevation of Mitochondrial Translation Genes

To determine whether SIRT7 is directed by NRF-1 to silence mitochondrial translation genes, we generated two SIRT7 KD cell lines using infection of two separate shRNA constructs followed by puromycin selection. We found that similar to ribosomal subunits, a large number of genes involved in mitochondrial translation are upregulated by knockdown of SIRT7 (Fig. 3.3 and Fig. 3.4B). Next, we determined whether upregulation of the mitochondrial genes is dependent on NRF-1 by knocking down NRF-1 in conjunction with SIRT7. We found that the upregulation of mitochondrial genes (GFM2 and MRPL24) was abrogated by KD of NRF-1 (Fig. 3.4A). As a control, the upregulation of RPS20, which is a non-mitochondrial target of SIRT7, was not abrogated by NRF-1 KD (Fig. 3.4A). Via qRT-PCR, we found that KD of NRF-1 was able to rescue the elevated expression of all SIRT7-targeted mitochondrial ribosomal subunits (Fig. 3.4B). Our results suggest that analogous to the situation with Myc and ribosomal subunits, NRF-1 promotes transcription of mitochondrial translation genes and mitochondrial ribosomal subunits unless SIRT7 binds to NRF-1 and mediates silencing of transcription.

SIRT7 is Upregulated by mtUPR via ATF5

After establishing the connection between SIRT7, NRF-1, and the transcription of mitochondrial translation genes, we next determined when SIRT7 would be upregulated to suppress mitochondrial translation. Since the mtUPR results in restoration of homeostasis in a similar manner as the ER UPR response, we assayed whether SIRT7 could play a similar role for mitochondria as it does for the ER. Inducers of mtUPR are not as well established as the inducers of ER stress. However, doxycycline, ethidium bromide, and paraquat have all been used as sources of mitochondrial stress through their ability to disturb mitochondrial protein homeostasis (Houtkouper *et al.*, 2013; Martinus *et al.* 1996). We treated cells with doxycycline (Fig. 3.5 A and Fig. 3.5D), ethidium bromide (Fig. 3.5B and Fig. 3.5C), and paraquat (Fig. 3.5E), and found that all treatments were able to induce the expression of SIRT7.

We next asked which transcription factor is responsible for upregulation of SIRT7 during mitochondrial stress conditions. We performed a bioinformatic analysis and found that SIRT7 has a conserved binding motif for activating transcription factor 5 (ATF5) in its promoter (Fig. 3.6A). A previous study has shown that the *C. elegans* homolog of ATF5 plays a crucial role in the mtUPR (Haynes *et al.*, 2010). Using a luciferase reporter assay, we found that ATF5 is able to activate transcription driven by the SIRT7 promoter (Fig. 3.6B). To show that SIRT7 induction is dependent on ATF5, we knocked down ATF5 and found that SIRT7 induction was significantly reduced upon treatment with ethidium bromide (Fig. 3.6C). Thus, SIRT7 induction upon mitochondrial stress is likely to be at least partially dependent on the activity of ATF5.

SIRT7-deficient Cells have More Severe Mitochondrial Stress

To determine the significance of SIRT7 for the mtUPR, we knocked down SIRT7 and measured the expression of the genes ClpP, HSP10, HSP60, and mtDnaJ, which have all been shown to be induced by mitochondrial stress (Pellegrino *et al*., 2013). In SIRT7 KD cell lines, we found elevated expression of these mitochondrial stress genes at both the protein (Fig. 3.7A) and mRNA level (Fig. 3.7B). We also found that treatment of SIRT7 KD cells with a truncated form of ornithine transcarbamylase (ΔOTC), which is a constitutively unfolded mitochondrial protein that leads to mitochondrial stress (Zhao *et al.* 2002), or ethidium bromide led to more severe mitochondrial stress than in control cells (Fig. 3.8A and Fig. 3.8B). Severe prolonged mitochondrial stress has been shown to lead to apoptosis. We subjected control and SIRT7 KD cell lines to prolonged ethidium bromide treatment, which depletes the cells of mtDNA and leads to mitochondrial stress. SIRT7 KD cells were significantly more prone to apoptosis than control cells in this assay (Fig. 3.9), suggesting that in the absence of SIRT7, cells are less able to manage mitochondrial stress.

NRF-1 KD Rescues Increased Mitochondrial Stress in SIRT7 KD Cells

Having found increased mitochondrial stress in SIRT7 KD cells, we next addressed whether the increased mitochondrial stress is due to the inability of NRF-1-dependent transcription to be silenced. We knocked down NRF-1 in SIRT7 KD cells and assayed the level of mitochondrial stress. We found that increased levels of the mitochondrial stress genes ClpP, HSP10, HSP60, and mtDnaJ in SIRT7 KD cells were rescued by NRF-1 KD (Fig. 3.10A and Fig. 3.10B). Importantly, the elevated expression of the ER stress marker grp78 was not rescued by NRF-1, suggesting that knocking down NRF-1 specifically rescues increased mitochondrial stress in SIRT7 KD cells.

SIRT7 KD Cells are More Prone to Nutrient Starvation-Induced Cell Death

After establishing the mechanistic link between SIRT7, NRF-1, and control of mitochondrial proteostasis, we explored physiological implications of this finding. Significant changes in mitochondrial morphology have been observed with nutrient starvation (Rambold *et al.* 2011), but the role of mitochondrial stress in this process has been poorly studied. SIRT7 expression is induced by glucose starvation and SIRT7 KD cells (Fig. 3.11A) have significantly worse survival under glucose starvation (Fig. 3.11B). SIRT7 KD cells also had worse survival under glutamine starvation (Fig. 3.11C), but the difference was not as severe as with glucose starvation.

The increased cell death in the SIRT7 KD cells could possibly have been due to their inability to shut down mitochondrial activity. It is possible that SIRT7 expression is activated by nutrient starvation to suppress NRF-1 dependent transcription of certain mitochondrial genes. To test for this possibility, we performed NRF-1 KD in SIRT7 KD cells, which led to partial rescue of survival in the KD cells under both glucose (Fig. 3.12A) and glutamine starvation (Fig. 3.12B). Thus, SIRT7 may play an important function in situations where mitochondrial activity needs to be suppressed.

SIRT7 Improves Hematopoietic Stem Cell Function by Combating Mitochondrial Stress

Rapid mitochondrial biogenesis has been shown to lead to mitochondrial stress (Pellegrino *et al.*, 2013). In the hematopoietic system, hematopoietic stem cells (HSCs) mostly reside in an oxygen-poor niche, where they are quiescent and rely mostly on glycolysis for energy. However, when they are called upon to replenish the hematopoietic system, HSCs undergo rapid proliferation and switch to a greater utilization of oxidative phosphorylation for energy (Suda *et al.*, 2011). This requires greater reliance on mitochondria, and the switch between quiescence and proliferation has been shown to lead to an increase in mitochondrial biogenesis (Romero-Moya *et al*., 2013). Given this background, we were interested in whether SIRT7 could improve HSC function by ameliorating

the mitochondrial stress that occurs with the transition between quiescence and proliferation.

We isolated HSCs from mice and cultured them in media rich in cytokines and growth factors to induce proliferation. The culturing process led to a significant increase in proliferation in the HSCs as indicated by increased Ki67 (Fig. 3.13A). The increased proliferation was accompanied by an increase in mitochondrial mass as assayed by the mitotracker green (MTG) dye (Fig. 3.13B). The proliferating HSCs also had a marked increase in mitochondrial stress as evidenced by the increase in expression of mtUPR genes (Fig. 3.13C).

In analyzing HSCs from WT and SIRT7 KO mice, we found increased mitochondrial stress in SIRT7 KO HSCs, which was abrogated by reintroduction of SIRT7 into the HSCs via lentiviral transduction (Fig. 3.14A). Reintroduction of SIRT7 also led to an increase in the colony-forming ability of SIRT7 KO HSCs in the colony forming cell (CFC) assay (Fig. 3.14B). Consistent with the mechanism that was worked out in cell lines, we found that KD of NRF-1 via lentiviral transduction was able to reduce mitochondrial stress in SIRT7 KO HSCs (Fig. 3.15A). KD of NRF-1 also led to an increase in the ability of SIRT7 KO HSCs to form colonies in the CFC assay (Fig. 3.15B).

SIRT7 Combats Mitochondrial Stress in Aged HSCs and Improves Function

As sirtuins are conserved longevity regulators (Finkel *et al.* 2009), our lab was interested in whether mitochondrial stress accumulates with age and whether SIRT7 could be used to combat this increase. We isolated HSCs from young (2-4 months) and aged (18-24 months) mice and found that aged HSCs had a marked increase in mitochondrial stress as assayed by the expression of mtUPR genes (Fig. 3.16). To determine if SIRT7 could decrease the mitochondrial stress in aged HSCs and improve their function, we overexpressed SIRT7 in the HSCs using lentiviral transduction and found that mitochondrial stress genes were reduced (Fig. 3.17A). Additionally, we found that overexpression of SIRT7 improved the function of aged HSCs. as evidenced by an increase in the colonies formed in the CFC assay (Fig. 3.17B).

Discussion

A New Component of the mtUPR

My work has extended the role of SIRT7 from the ER stress response to the response to mitochondrial stress. Through treating cells with mtUPR inducers, we have found SIRT7 expression to be upregulated in response to the stress with at least part of the transcriptional regulation of SIRT7 being due to ATF-5. We have also found that SIRT7 binds to NRF-1 and suppresses a subset of its target genes involved in mitochondrial translation. In this manner, SIRT7 is able to alleviate mitochondrial stress, possibly by slowing translation to buy time to allow mitochondrial-specific chaperones to properly fold misfolded mitochondrial proteins. Since SIRT7 regulates both general translation and

mitochondrial-specific translation, it is also possible that SIRT7 can coordinate the proper stoichiometric ratio of nuclear and mtDNA-encoded mitochondrial proteins, which has been shown to lead to mitochondrial stress when out of balance (Houtkooper *et al.* 2013).

Implications for HSC Function and Aging

As HSCs dynamically regulate their mitochondrial activity and mass in response to quiescence and proliferation, they must be able to cope with changing demands on the mitochondrial translation and protein folding apparatus. Through characterizing the HSCs of SIRT7 KO mice, our lab has found them to have higher levels of mitochondrial stress and harbor defects in quiescence, reconstitution, and differentiation. Overexpression of SIRT7 or KD of NRF-1 was effective in alleviating the increased mitochondrial stress in SIRT7 KO HSCs and improved their function.

We have also shown that aged HSCs have increased mitochondrial stress and that alleviating the mitochondrial stress through overexpression of SIRT7 can improve their function. A study has shown that the expression of SIRT7 decreases with age in HSCs (Chambers *et al.* 2007). It is possible that the decline in SIRT7 with age may lead to compromised resistance to mitochondrial stressors. This leads to increased mitochondrial stress in aged HSCs, possibly contributing to their decline in function.

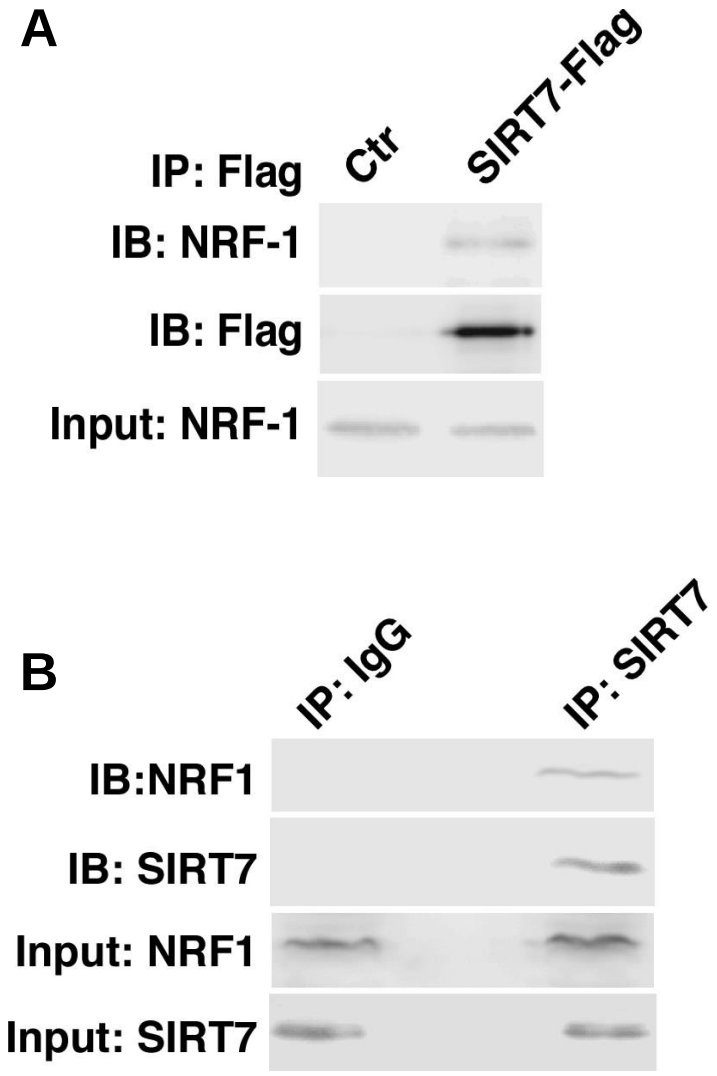


Fig. 3.1: SIRT7 interacts with NRF1
(A-B) Western blot showing co-immunoprecipitation of Flag-tagged SIRT7 (A) and endogenous SIRT7 (B) with endogenous Myc in 293T cells.

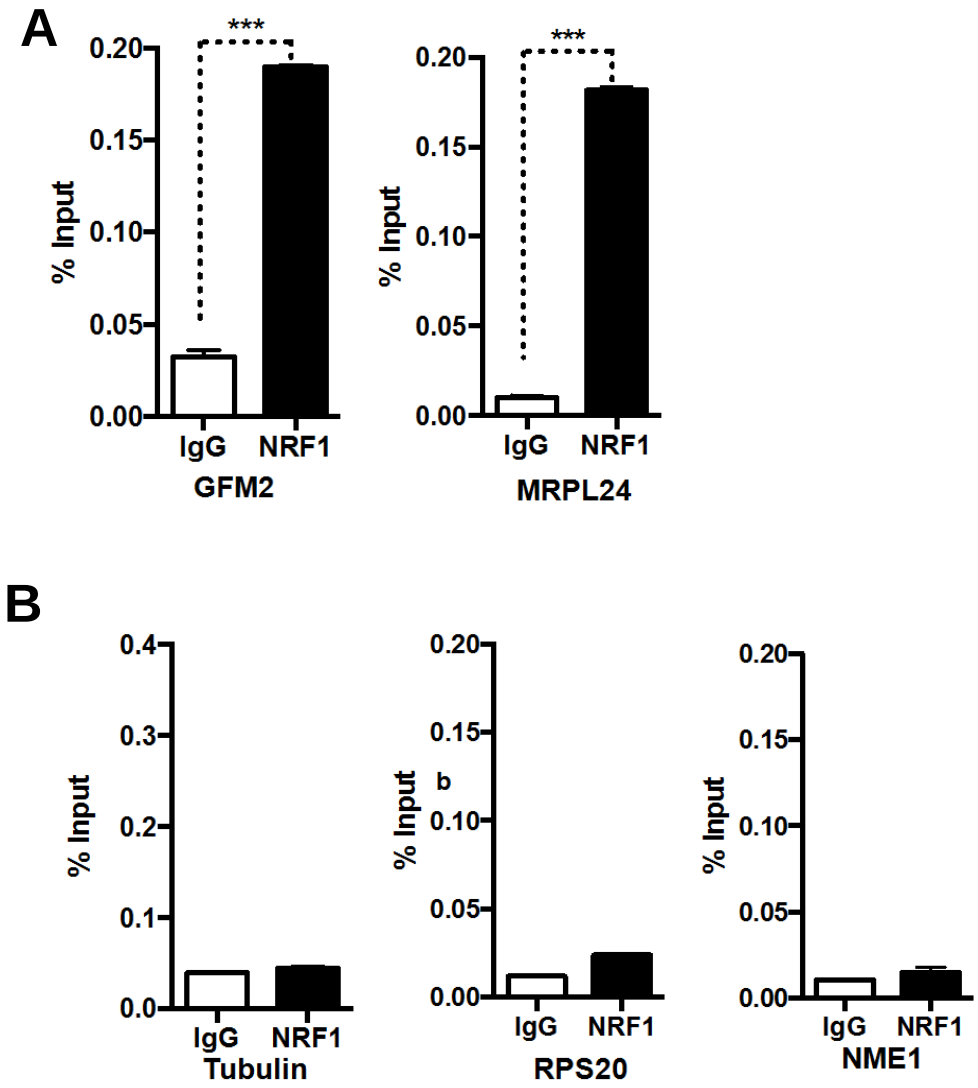


Fig. 3.2: NRF-1 is enriched at SIRT7 mitochondrial targets but not other known SIRT7 targets

(A-B) ChIP qPCR showing NRF-1 occupancy at the same regions of the proximal promoters as SIRT7 at SIRT7 mitochondrial target genes (A), but not the proximal promoters of a control gene (tubulin) or other SIRT7 targets (RPS20 and NME1) (B).

Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

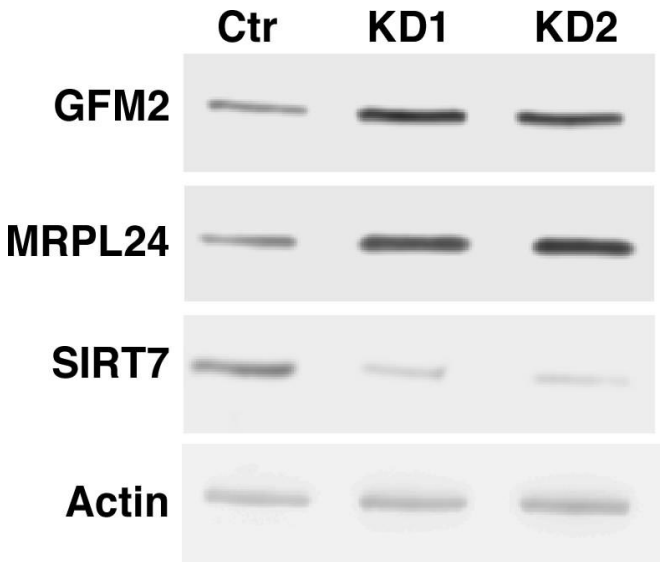


Fig. 3.3: Mitochondrial translation genes are upregulated in SIRT7 KD cells
Western blot showing the upregulation of mitochondrial translation genes (GFM2 and MRPL24) in SIRT7 KD cells.

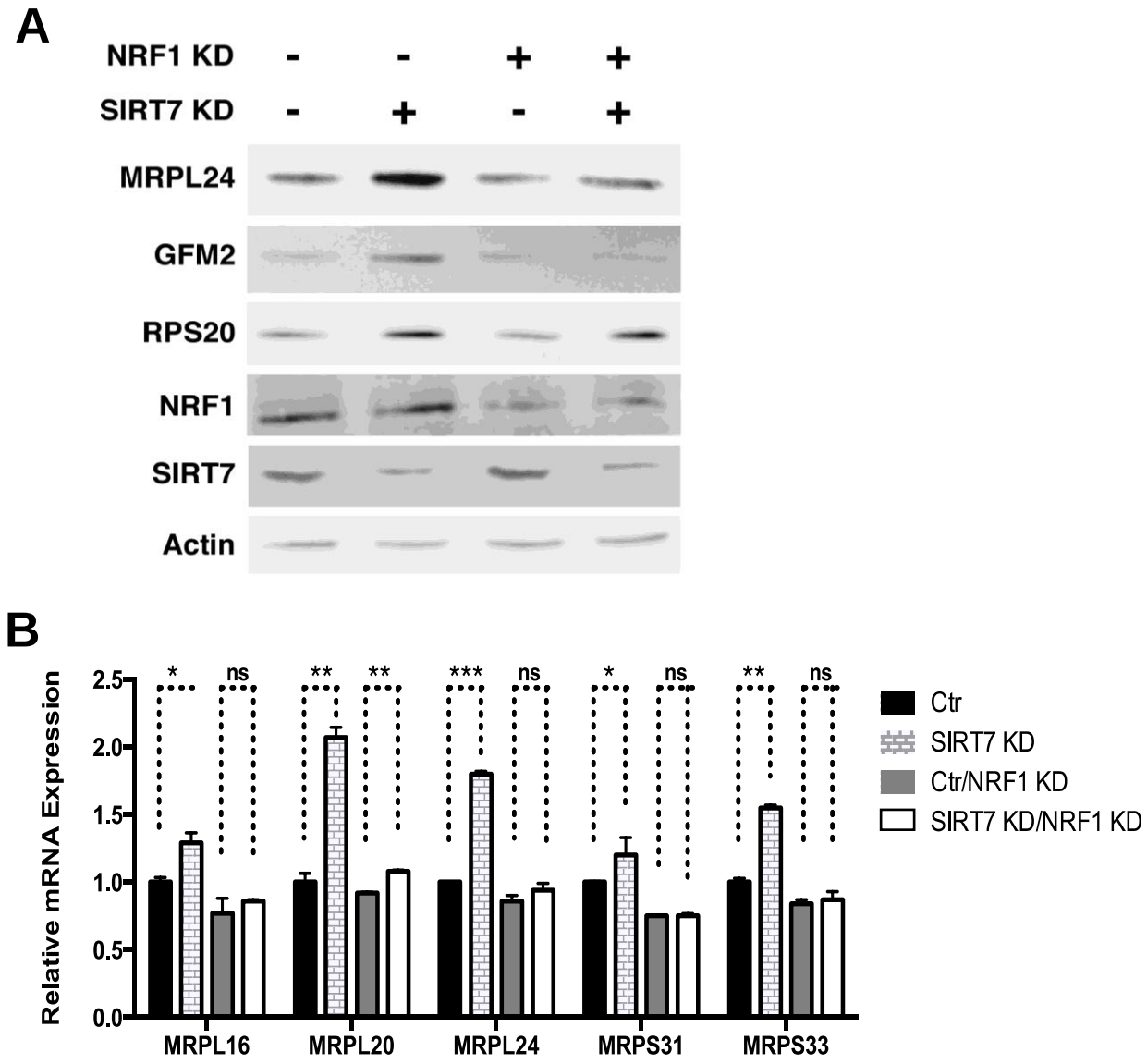


Fig. 3.4: Elevation of mitochondrial ribosomal genes in SIRT7 KD cells is rescued by KD of NRF-1

(A) Western blot showing that KD of NRF-1 rescues the increased expression of mitochondrial translation genes (GFM2 and MRPL24), but not RPS20, in SIRT7 KD cells.

(B) KD of NRF-1 rescues the increased expression of mitochondrial ribosomal proteins in SIRT7 KD cells.

Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

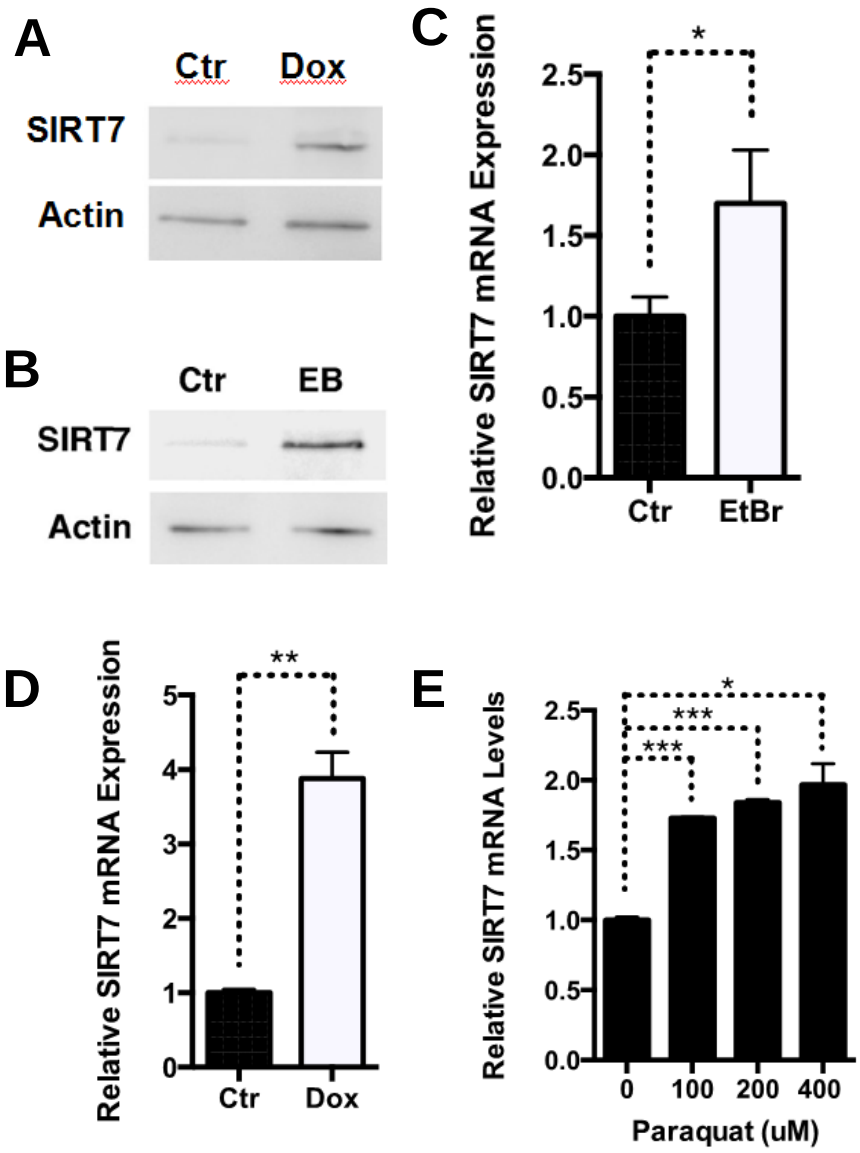


Fig. 3.5: SIRT7 is induced by treatments that induce mitochondrial stress
(A-B) Western blots showing increased levels of SIRT7 after treatment of 293T cells with the mitochondrial UPR stress inducers doxycycline (A) or EtBr (B).
(C-D) Quantitative RT-PCR showing increased expression of SIRT7 mRNA upon treatment of 293T cells with the mitochondrial UPR stress inducers ethidium bromide (C) or doxycycline (D).
(E) Quantitative RT-PCR showing increased expression of SIRT7 upon treatment of 293T cells with increasing levels of the mitochondrial stress inducer paraquat. Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

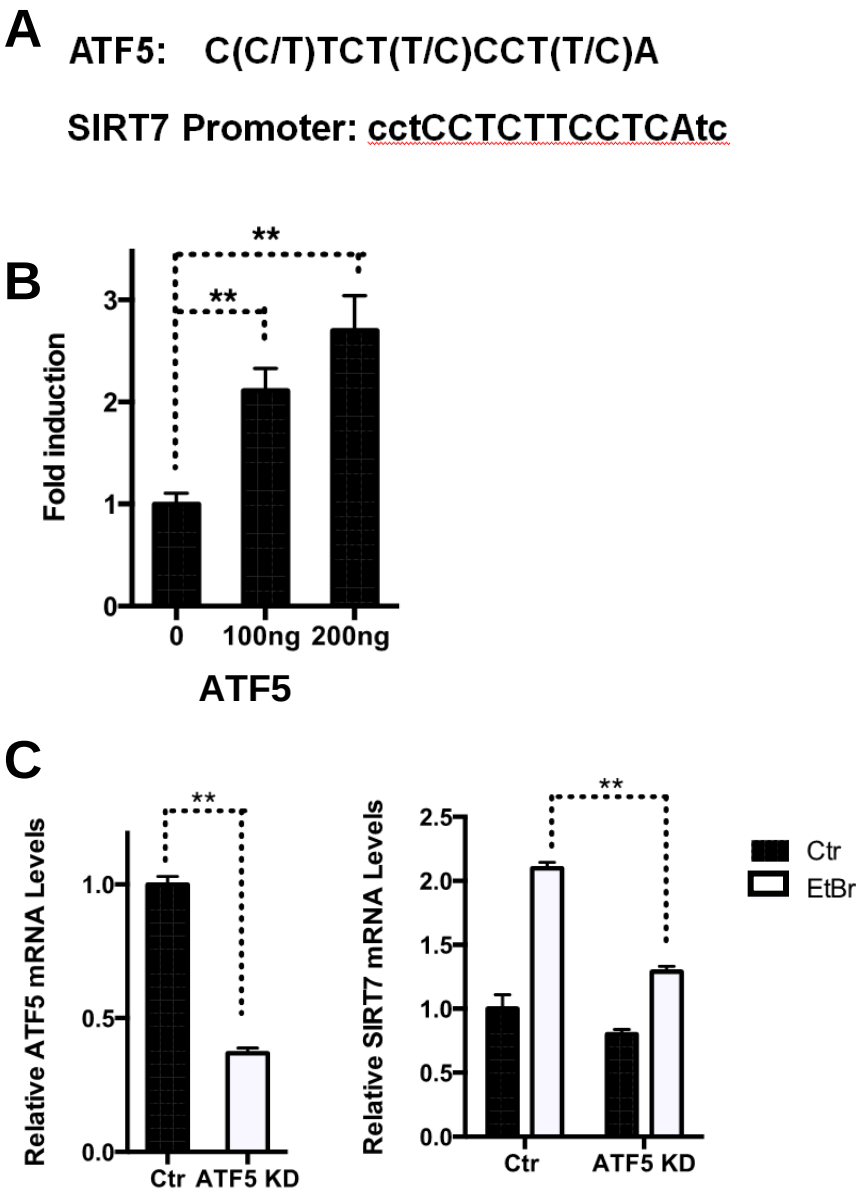


Fig. 3.6: SIRT7 induction by mitochondrial stress is dependent upon ATF5
(A) Bioinformatic analysis showing the presence of the ATF5 consensus binding sequence in the SIRT7 promoter.
(B) Luciferase assay showing increasing transcription driven by the SIRT7 promoter upon increasing levels of ATF5.
(C) Quantitative RT-PCR showing knockdown of ATF5 and decreased induction of SIRT7 by ethidium bromide after knockdown of ATF5.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

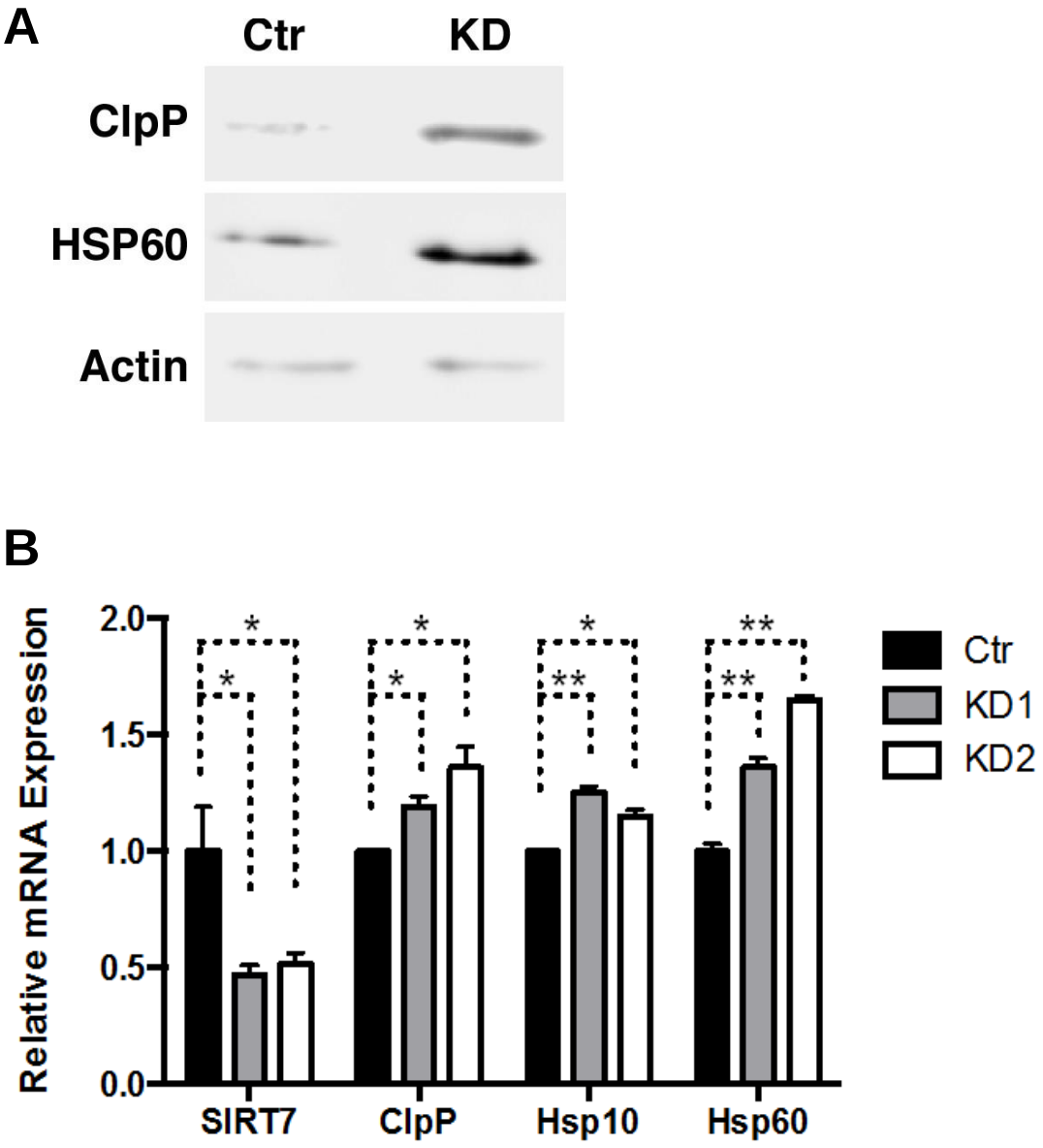


Fig. 3.7: SIRT7 KD cells have constitutive mitochondrial stress
(A) Western blot showing increased expression of mitochondrial UPR stress genes (ClpP and HSP60) upon KD of SIRT7.
(B) Quantitative RT-PCR showing increased expression of mitochondrial UPR stress genes (ClpP, Hsp10, and Hsp60) upon KD of SIRT7l.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

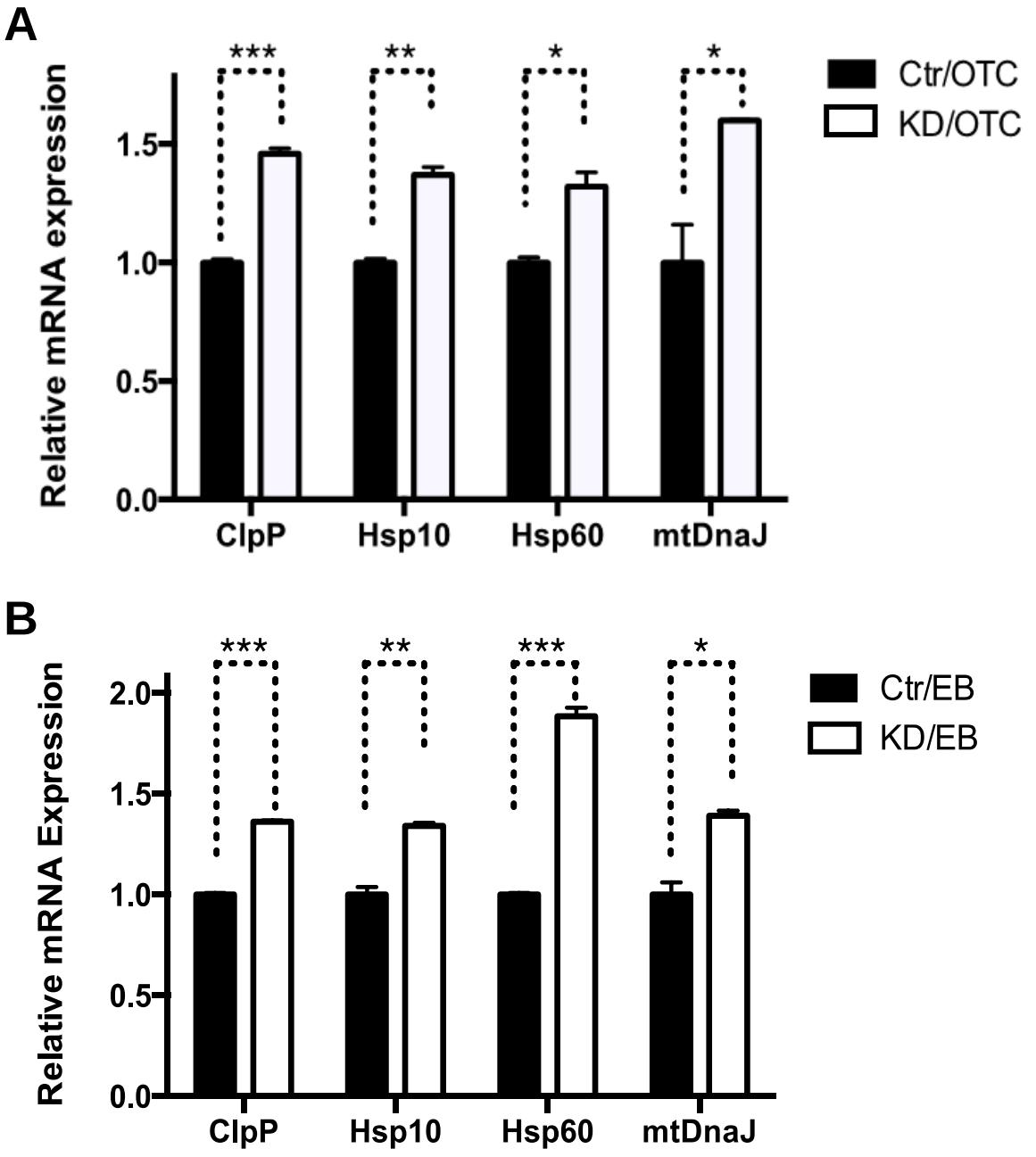


Fig. 3.8: SIRT7 KD cells have higher mitochondrial stress than control cells when treated with mtUPR inducers

(A-B) Quantitative RT-PCR showing increased expression of mitochondrial UPR genes in SIRT7 KD cells relative to control cells after treatment of cells with the mitochondrial UPR stress inducers OTC (A) or ethidium bromide (B).
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

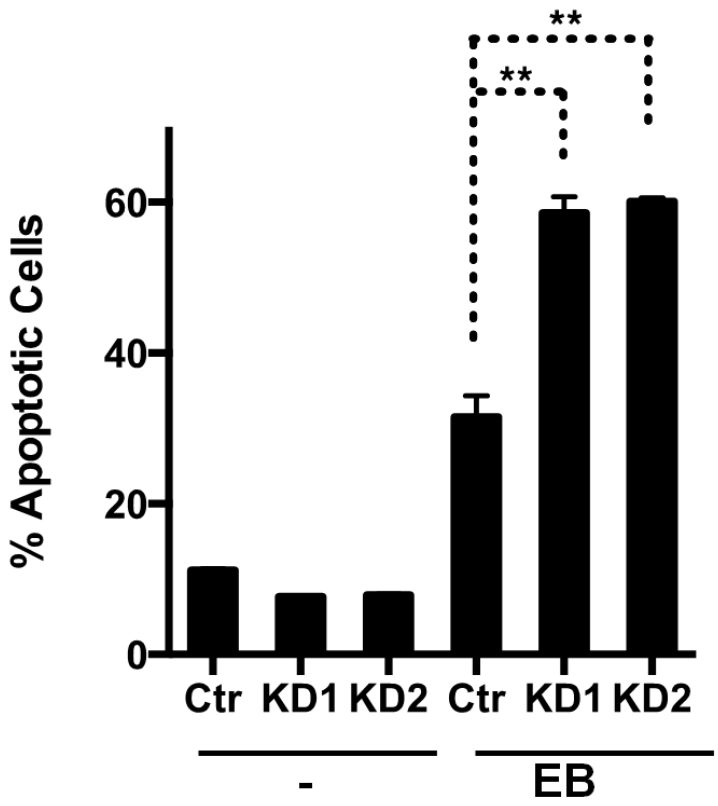


Fig. 3.9: SIRT7 KD cells are more prone to mtUPR-induced apoptosis
Annexin V staining showing increased apoptosis in SIRT7 KD cells upon induction of chronic mitochondrial UPR stress using ethidium bromide. Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

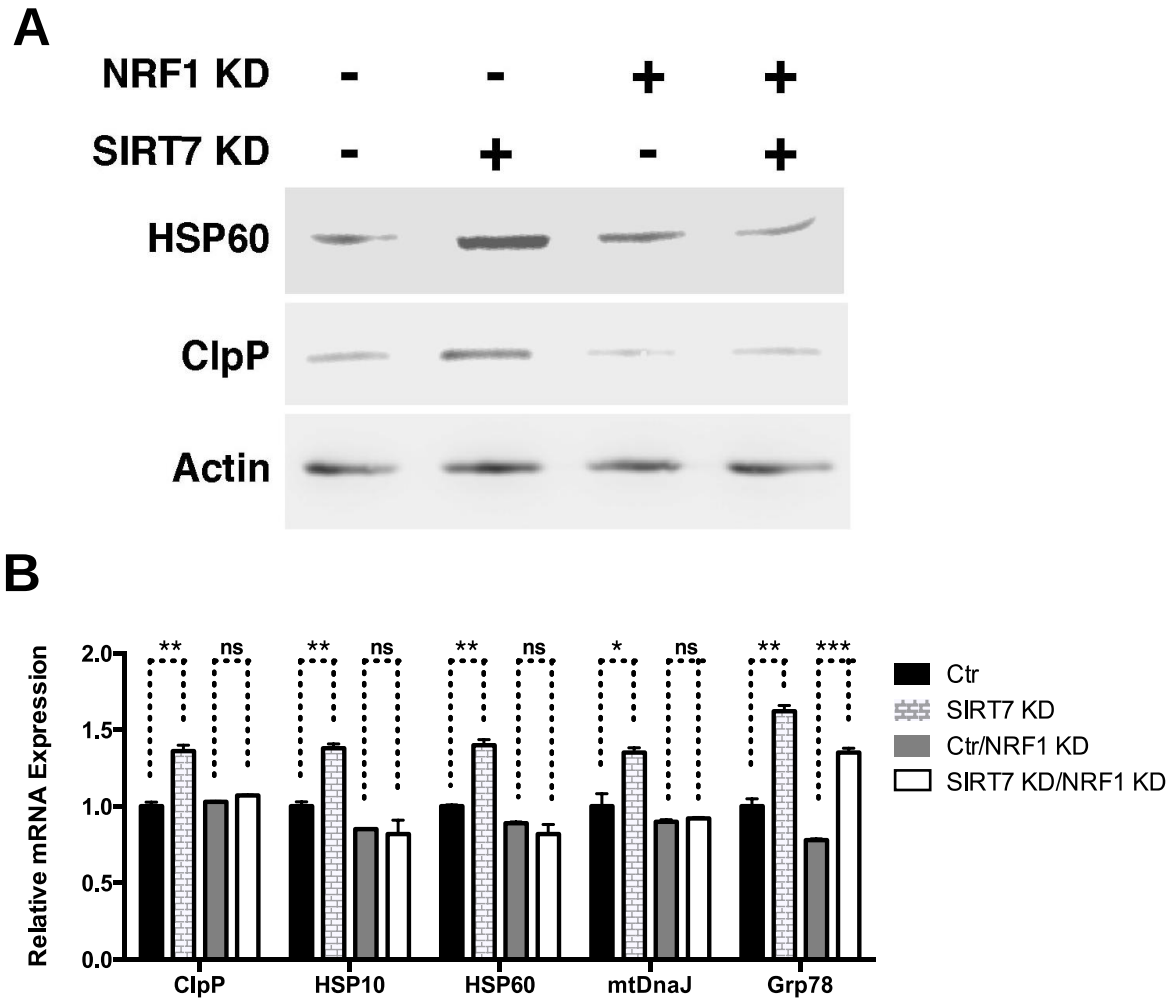


Fig. 3.10: NRF-1 KD rescues the elevated mitochondrial stress in SIRT7 KD cells

(A) Western blot showing that KD of NRF-1 rescues the elevation of mitochondrial UPR stress genes in SIRT7 KD cells.

(B) Quantitative RT-PCR showing that KD of NRF-1 rescues the elevation of mitochondrial UPR stress genes, but not the ER stress gene Grp78, in SIRT7 KD cells.

Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

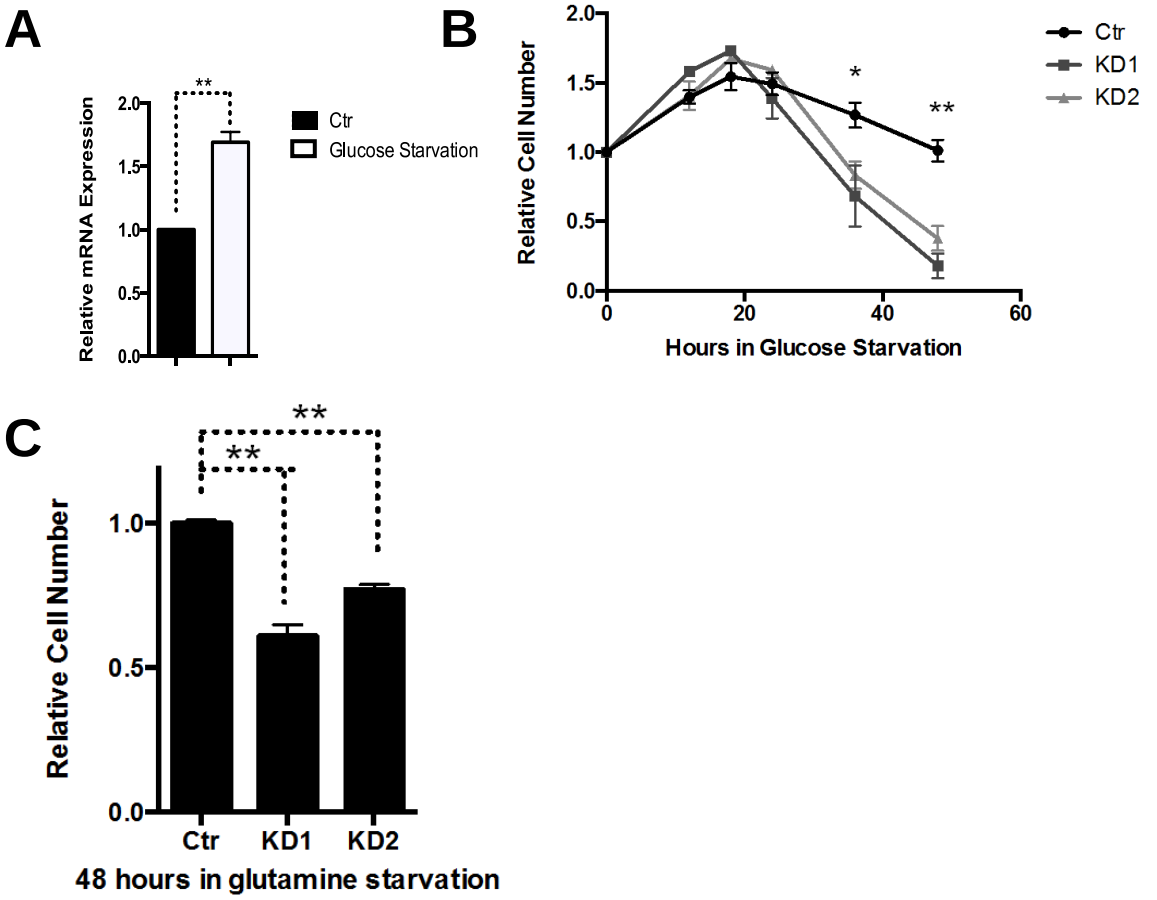


Fig. 3.11: SIRT7 KD cells are more prone to nutrient starvation-induced cell death

(A) Quantitative RT-PCR showing induction of SIRT7 expression upon placement of 293T cells into media without glucose.

(B) Growth curve showing decreased survival of stable SIRT7 KD 293T cells compared to control cells upon glucose starvation.

(C) Bar graph showing decreased survival of stable SIRT7 KD 293T cells compared to control cells upon 48 hours of glutamine starvation

Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

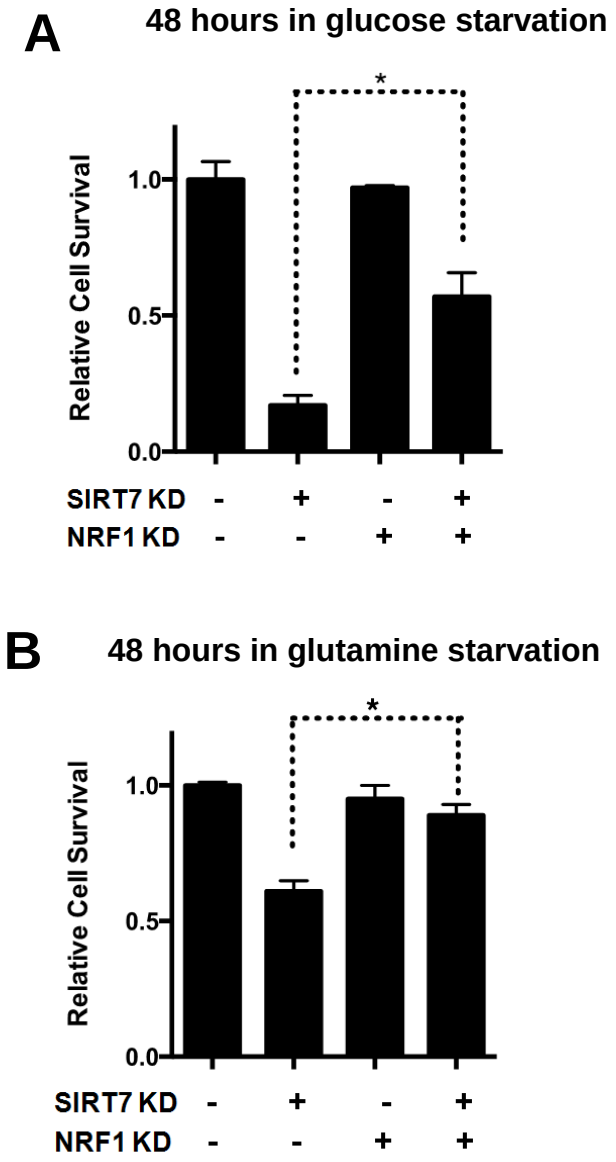


Fig. 3.12: NRF1 KD rescues sensitivity of SIRT7 KD cells to nutritional starvation
(A-B) KD of NRF-1 improves the survival of SIRT7 KD cells that are subject to glucose (A) or glutamine (B) starvation.
Error bars represent SEM: *: p<0.05, **: p<0.01, ***: p<0.001, ns: p>0.05

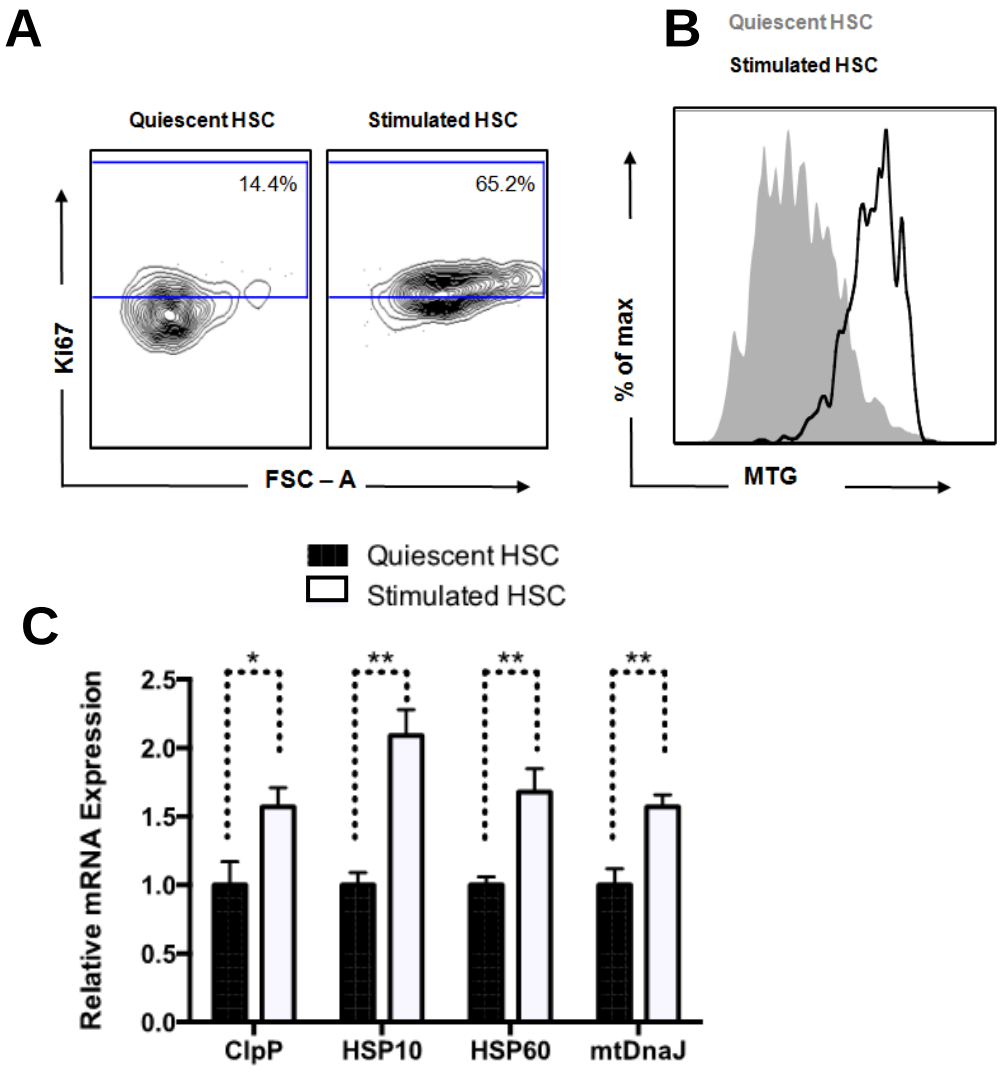


Fig. 3.13: Proliferating HSCs have increased mitochondrial mass and mitochondrial stress

(A) FACs contour plot of HSCs using Ki-67 staining showing increased proliferation in HSCs stimulated by ex-vivo culture with cytokines compared to quiescent HSCs freshly isolated from mouse bone marrow.

(B) FACs plot showing higher mitotracker green (MTG) staining in HSCs stimulated by ex-vivo culture compared to quiescent HSCs freshly isolated from mouse bone marrow.

(C) Quantitative RT-PCR showing increased expression of mitochondrial UPR stress genes in HSCs stimulated by ex-vivo culture compared to quiescent HSCs freshly isolated from mouse bone marrow.

Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

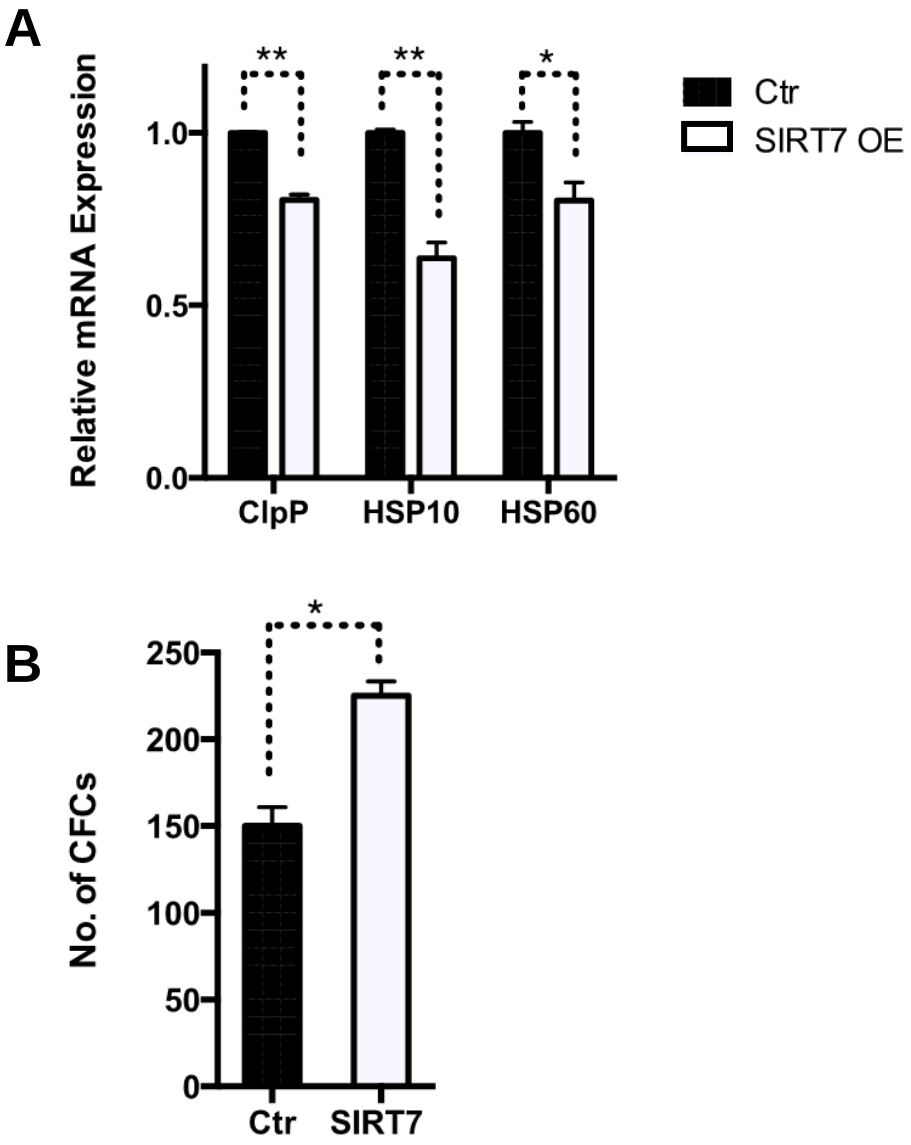


Fig. 3.14: SIRT7 overexpression in SIRT7 KO HSCs decreases mitochondrial stress and improves colony formation ability
(A) Quantitative RT-PCR showing that overexpression of SIRT7 via lentiviral transduction in SIRT7 KO HSCs leads to reduced expression of mitochondrial UPR stress genes.
(B) Colony forming cell (CFC) assay showing that overexpression of SIRT7 via lentiviral transduction in SIRT7 KO HSCs leads to increased formation of colonies.
Error bars represent SEM: *: p<0.05, **: p<0.01, ***: p<0.001, ns: p>0.05

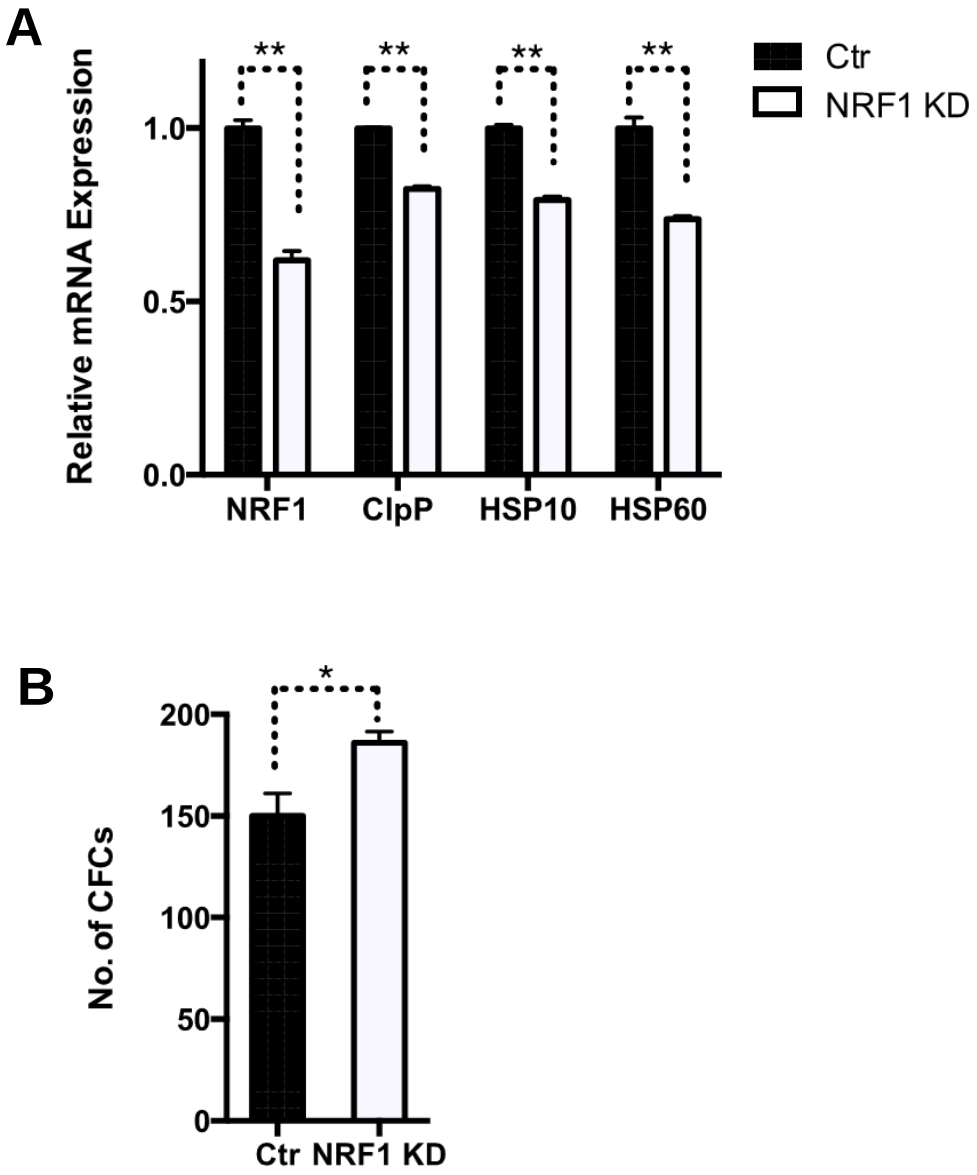


Fig. 3.15: NRF-1 KD reduces mitochondrial stress in SIRT7 KO HSCs and improved colony formation ability
(A) Quantitative RT-PCR showing that KD of NRF-1 via lentiviral transduction in SIRT7 KO HSCs leads to reduced expression of mitochondrial UPR stress genes.
(B) Colony forming cell (CFC) assay showing that KD of NRF-1 via lentiviral transduction in SIRT7 KO HSCs leads to increased formation of colonies.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

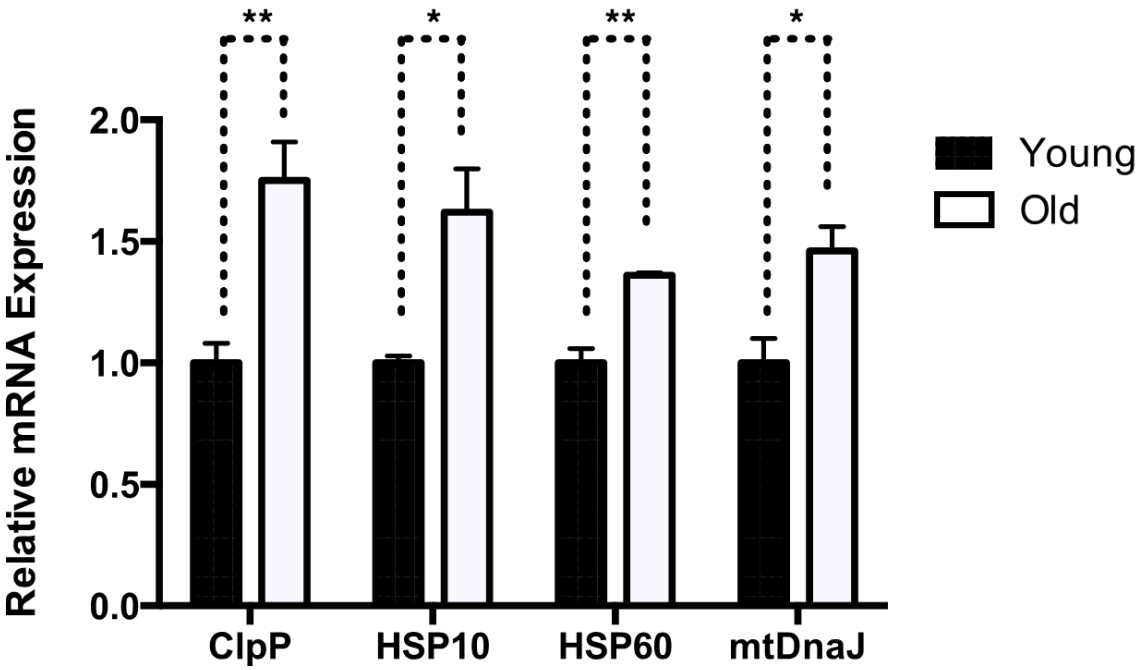


Fig. 3.16: Aged HSCs have increased mitochondrial stress
Quantitative RT-PCR showing increased expression of mitochondrial UPR stress genes in HSCs from aged mice (18-24 months) compared to HSCs from young mice (2-4 months).
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

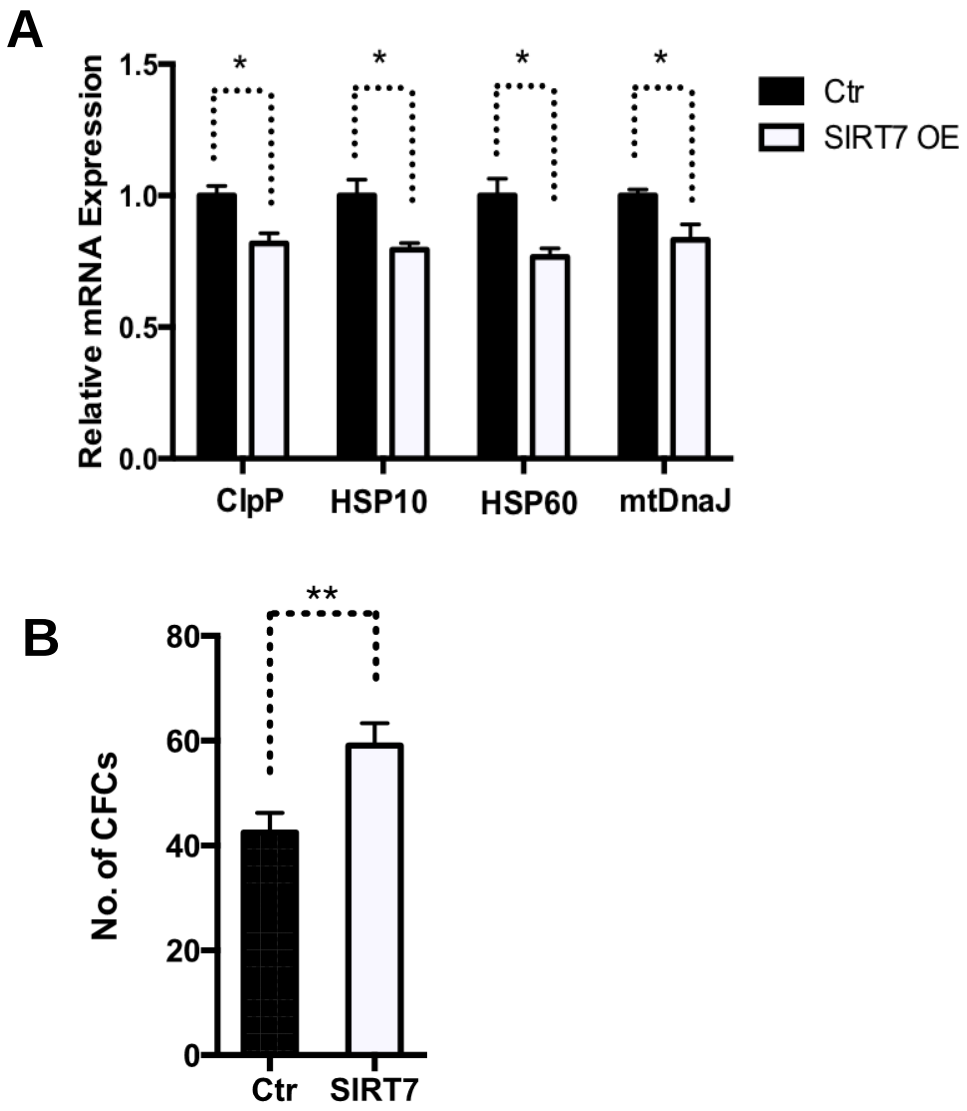


Fig. 3.17: SIRT7 overexpression reduces mitochondrial stress and improves colony formation ability in aged HSCs
(A) Quantitative RT-PCR showing that overexpression of SIRT7 via lentiviral transduction in HSCs from aged mice leads to reduced expression of mitochondrial UPR stress genes.
(B) Colony forming cell (CFC) assay showing that overexpression of SIRT7 via lentiviral transduction in HSCs from aged mice leads to increased formation of colonies.
Error bars represent SEM: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: $p>0.05$

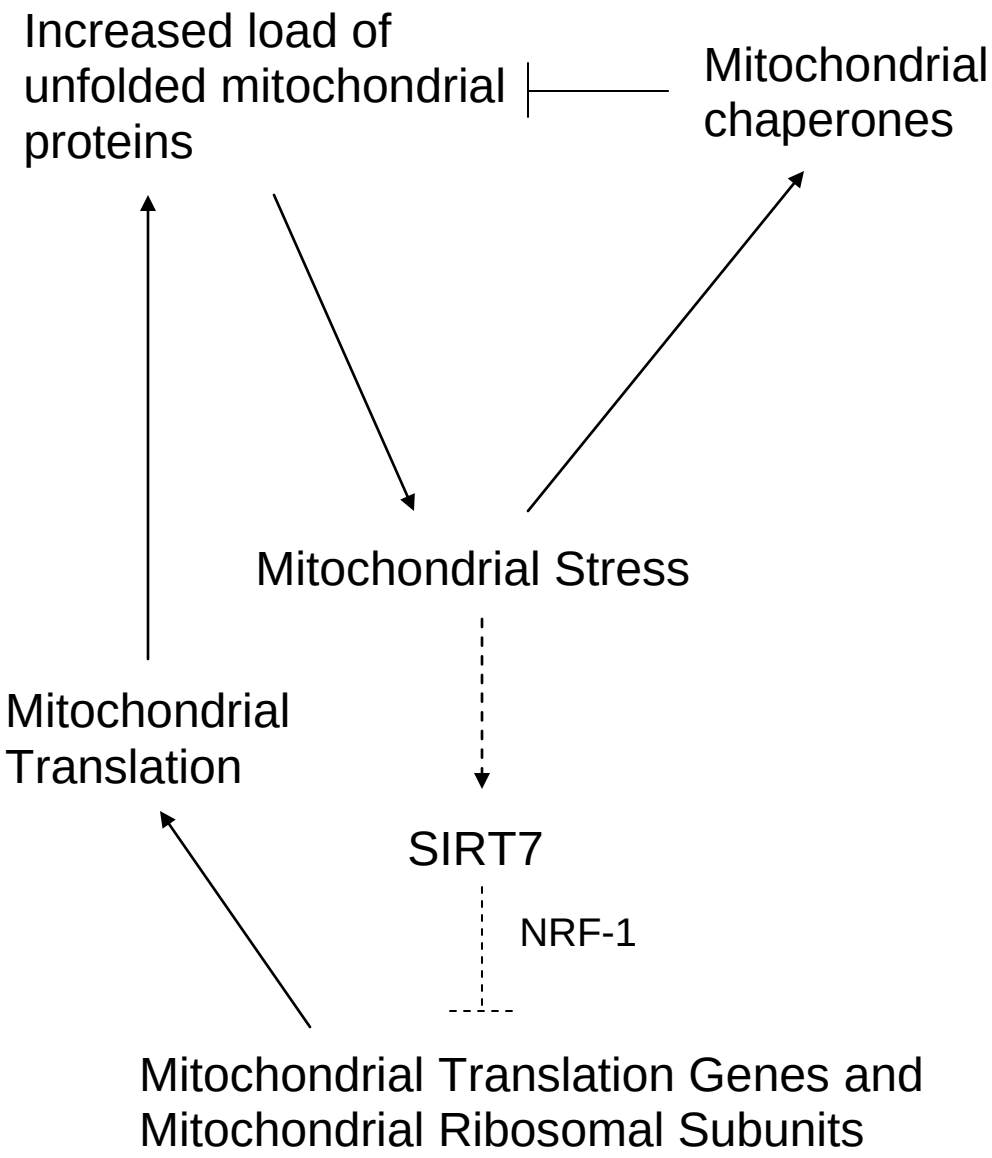


Fig. 3.18: Model of the role of SIRT7 in management of mitochondrial stress Increased load of unfolded mitochondrial proteins leads to mitochondrial stress, which triggers expression of mitochondrial chaperones, alleviating the stress. We propose that mitochondrial stress also increases the expression of SIRT7, which binds to NRF-1 and inhibits the expression of mitochondrial translation genes and mitochondrial ribosomal subunits (dotted lines), which decreases mitochondrial translation, and alleviates mitochondrial stress.

Chapter 4

Future Directions and Closing Remarks

Specificity of the SIRT7 Response to Stress

Our work has shown that SIRT7 plays an important role in both the ER stress response and the mitochondrial UPR. Stressors that induce both types of stress increase the expression of SIRT7, which binds to the promoters of a subset of ribosomal subunit genes in the case of ER stress and mitochondrial translation genes in the case of mitochondrial stress. At the promoters, SIRT7 deacetylates H3K18, which leads to compaction of chromatin and silencing of gene expression. Lacking a DNA binding domain, SIRT7 is directed to its target genes by Myc for ribosomal subunits and NRF-1 for mitochondrial translation genes. By silencing ribosomal subunits, SIRT7 decreases translation, which allows for restoration of homeostasis during ER stress conditions. Analogously, by silencing mitochondrial translation genes, SIRT7 allows for restoration of mitochondrial homeostasis during conditions of mitochondrial stress.

A major question that remains to be addressed is the specificity of SIRT7 binding to its target genes. While SIRT7 is directed to its targets by Myc and NRF-1, it only binds to a subset of Myc and NRF-1 targets. A few possibilities to explain this finding is that the promoters of SIRT7 targets have motifs that are specific to SIRT7-Myc or SIRT7-NRF-1, only the SIRT7 target genes are strongly regulated via H3K18 acetylation, or SIRT7 binds to one or more adapter proteins that confer specificity to a subset of Myc or NRF-1 targets.

To test the first possibility, a bioinformatic analysis of SIRT7-Myc or SIRT7-NRF-1 target promoters could be performed and sequences unique to SIRT7 targets versus other Myc or NRF-1 targets could be identified. A structural analysis of the complex formed by SIRT7-Myc or SIRT7-NRF-1 could be performed to determine the rationale for binding to predicted consensus sequences.

For the second possibility, it is possible that only a certain subset of Myc and NRF-1 targets are strongly regulated by H3K18 acetylation. The acetylation of H3K18 may be required for SIRT7 to dock at the promoters and remove the acetyl group. As a first step to test this hypothesis, ChIP can be used to determine whether H3K18 acetylation is highest at SIRT7 target genes and lower at other Myc and NRF-1 targets under basal conditions.

For the third possibility, finding interacting partners of SIRT7 may help answer another major question related to SIRT7 function, which is how its localization is regulated. SIRT7 was originally found to be mostly localized to the nucleolus, where it promotes rDNA transcription (Ford *et al.* 2006). However, recent studies, including the work presented in this dissertation indicate that SIRT7 functions in the nucleus as well, where it binds to chromatin and silences the expressions of numerous ribosomal and mitochondrial genes (Barber *et al.*

2012, Shin *et al.* 2013). Therefore, we must come to a better understanding of the conditions under which SIRT7 promotes rDNA transcription versus mediating repressive effects via chromatin.

One possibility is that SIRT7, like many other nucleolar proteins, functions as a stress sensor. They are localized to the nucleolus unless certain stresses occur, which leads to translocation to the nucleus or cytoplasm. This has been shown to be an important response to oncogenic stress (p19) (Zhang *et al.*, 1998) and nucleolar stress (ribosomal subunits (Zhang and Lu, 2009) and MYBBP1A (Ono *et al.* 2014)). However, thus far, nucleolar translocation has not been associated with ER stress or mitochondrial stress. SIRT7 has been shown to relocalize from the nucleolus to the nucleoplasm under various stress conditions that impair rDNA transcription and disrupt the nucleolus (Chen *et al.*, 2013). It is possible that nucleolar disruption occurs in response to ER stress and mitochondrial stress, which then allows nucleolar components, such as SIRT7, to bind to Myc and NRF-1 in the nucleoplasm to specifically suppress general and mitochondrial translation to promote return to homeostasis.

NPM1 has been shown to interact with Myc (Li *et al.*, 2008), and we have shown that it is also pulled down by SIRT7. Meanwhile, RPL11 has been shown to translocate to the nucleoplasm under conditions of excessive ribosomal transcription and inhibit Myc specifically through inhibiting acetylation of the histone at target genes (Dai *et al.*, 2007). Lastly, MYBBP1A, which interacts with numerous transcription factors is a translocating nucleolar protein that has been shown to interact with SIRT7 (Karim *et al.*, 2013).

To test the SIRT7 translocation hypothesis, nucleolar structure and SIRT7 localization can be visualized under normal conditions and conditions that induce mitochondrial stress. If the hypothesis holds true, the nucleolus will break up and SIRT7 will be more diffusely spread over the nucleus under ER stress or mitochondrial stress conditions. One approach to determining the candidate interacting partners of SIRT7 is to knock them down one-by-one and determine whether SIRT7 can still bind to Myc or NRF-1 and its target genes. ChIP-seq can also be used to determine the global occupancy of these candidate interacting partners and determine how closely they overlap with SIRT7 occupancy.

As a future direction, understanding the specificity of the SIRT7 stress response holds the promise of not only advancing our knowledge of sirtuin biology, but could also bring a new conceptual understanding of the role of the nucleolus in the response to ER stress and mitochondrial stress.

SIRT7 and Longevity

Sirtuins have been shown to promote longevity in numerous model organisms. However, in mammals, the only sirtuin that has been shown to extend lifespan when overexpressed is SIRT6 (Kanfi *et al.* 2012). One mechanism that has been shown to promote lifespan extension is to increase the activity of an organism's natural defense against stress (Kapahi *et al.* 1999). Both ER stress

and mitochondrial stress, when mild, have been shown to promote lifespan extension by increasing the expression of chaperones and genes involved in stress resistance (Labunskyy *et al.* 2014, Houtkooper *et al.* 2013). Our work has shown that SIRT7 is one of the genes that is induced by both types of stresses. It will be interesting to determine whether any of the increase in longevity induced by these stresses is dependent on SIRT7. It would also be informative to generate a SIRT7 transgenic mouse and determine if it has a more robust response to ER stress and/or mitochondrial stress and whether this leads to lifespan extension.

My work has already shown that in the absence of SIRT7, there is chronic ER stress and mitochondrial stress, which is detrimental to the cell. The chronic ER stress and mitochondrial stress are also detrimental at the organismal level in the form of fatty liver and compromised HSC function respectively. A separate study using an independently derived line of SIRT7 KO mice has determined that they have shortened lifespans due to increased cardiomyopathy (Vakhrusheva *et al.* 2008). While deficiency of SIRT7 has been shown to lead to decreased stress resistance and even decreased longevity, the ultimate test for whether SIRT7 promotes longevity is the generation of a transgenic mouse and performing a Kaplan-Meyer lifespan curve.

The Connection Between SIRT7 and Cancer

The role of sirtuins in cancer has been controversial, as studies have shown that they have properties of both tumor suppressors and oncogenes (Bosch-Presegue and Vaquero 2011). A theme that has emerged from studies on sirtuins and cancer is that by protecting against cellular stress and damage, they act as tumor suppressors and prevent tumorigenesis. SIRT1 (Firestein *et al.* 2008), SIRT2 (Kim *et al.* 2011), SIRT3 (Kim *et al.* 2010), SIRT4 (Jeong *et al.* 2013), and SIRT6 (Sebastian *et al.* 2012) have all been shown to prevent tumorigenesis under physiological conditions. However, in established tumors, these same attributes of sirtuins protect cancer cells and promote cancer growth (Bosch-Presegue and Vaquero 2011). Thus, the role of sirtuins in cancer is likely to be context-dependent.

The relation of SIRT7 to cancer is similar to that of other sirtuins. Two studies have shown that SIRT7 promotes malignancy and growth in established tumors by inhibiting tumor suppressors (Barber *et al.* 2012, Kim *et al.* 2012). However, the role of SIRT7 in tumorigenesis may be very different. The connection established between SIRT7 and ER stress suggests that SIRT7 might be an intriguing target both in treating established cancers and in preventing tumorigenesis.

Cancer cells are known to require a robust UPR to deal with the extra load of proteins that they are producing in order to sustain rapid growth and proliferation. Due to this fact, treatments that compromise the UPR and lead to excess ER stress have been shown to be effective in inducing apoptosis in

cancer cells (Verfaillie *et al.*, 2013). Two studies have shown that targeting SIRT7 in established cancer cells leads to cell death with the proposed mechanism being upregulation of tumor suppressors (Barber *et al.* 2012, Kim *et al.* 2012). However, our finding of the role of SIRT7 in suppressing ER stress suggests that KD of SIRT7 may cause death in cancer cells through induction of ER stress. It will be interesting to target SIRT7 in cancer cells with high expression levels and determine if ER stress-induced apoptosis is occurring, which could form the basis for novel cancer therapies.

No published study has yet found SIRT7 to function as a tumor suppressor. However, my mechanistic work with SIRT7 suggests that it might harbor an anti-tumorigenic function. We have shown that SIRT7 inhibits Myc, which is one of the most commonly upregulated genes in cancers. To determine the implication of this finding for proliferation, we knocked down SIRT7 and found increased cell proliferation (Fig. 4.1A). Conversely, overexpression of SIRT7, but not catalytically inactive SIRT7 led to decreased proliferation in cells (Fig. 4.1B). We found that SIRT7's effect on proliferation is Myc-dependent as KD of Myc eliminated the difference in proliferation between SIRT7 KD and control cells (Fig. 4.2). Thus, with deficiency of SIRT7, there is more Myc-induced proliferation.

Additionally, our lab has found that SIRT7 KO mice have an increased tendency to develop liver tumors (Fig. 4.3). These results are not entirely unexpected as SIRT7 KO mice develop NAFLD due to increased ER stress (Shin *et al.* 2013). NALFD has been shown to lead to cirrhosis and in severe cases, hepatocellular carcinoma. By protecting against ER stress and associated inflammation and hepatocyte injury, SIRT7 is likely protecting against tumorigenesis. Similarly, smoking has been shown to lead to ER stress and inflammation in the lungs, which contributes to lung cancer development. It is possible that SIRT7, once again through protecting against ER stress, may also inhibit lung tumorigenesis.

Future studies will need to be done on how SIRT7 functions in tumorigenesis under physiological conditions. These could utilize conditional knockout or transgenic mice crossed to mice that are genetically susceptible to certain cancers or treated with chemical inducers of tumorigenesis. Given our findings on the role of SIRT7 in combating ER stress and suppressing a subset of Myc genes, it is quite likely that SIRT7 will be found to play a protecting role against the development of cancer.

Closing Remarks

When I started graduate school, my main objective was to add something, no matter how little, to our understanding of the molecular underpinnings of human disease. In the process, I hoped to learn what it took to be a good scientist. While exploring possible labs to join, I was attracted to my dissertation

lab by its research program in the molecular mechanisms underlying aging. Since many degenerative diseases are associated with the aging process, tackling aging held the promise of making dents in multiple diseases at once. I set out on my first big scientific odyssey armed with high hopes and mice that were lacking one the least understood members of the sirtuin family, SIRT7. Over the course of the last few years, my project has taken many twists, turns, and dead ends, which is not quite reflected in the linear progression of results presented in this dissertation.

With regards to my main objective, my expectations had to be tempered considerably as I realized how difficult it was to obtain not just positive results, but even results that were definitively negative. A lot of my data ended up in the fuzzy zone somewhere in between, which often made deciding on the next step very difficult. This made me realize the importance of designing well-controlled experiments that minimized ambiguity. Even more so, it made me realize the importance of being persistent, to distinguish between artifacts due to experimental design and actual biological results, and to keep trying until I could obtain data that was as clean as possible. Through this process, I feel that I have experienced tremendous growth as a scientist.

The verdict is still out for the significance of our work to human disease. We have made connections between SIRT7 and both ER stress and mitochondrial stress. In model organisms, increased abilities to cope with ER stress and mitochondrial stress have been linked to longevity. It is still unclear whether SIRT7 plays a role in regulating lifespan or health span, although based on our data, it seems promising that SIRT7 will be beneficial in this regard. Understanding the link between SIRT7, ER stress, and fatty liver disease opens up a potentially new way to target fatty liver disease. Finally, my work hints at the possibility that SIRT7 could function as a tumor suppressor under physiological conditions and migh be able to protect against cancers that develop due to chronic ER stress and inflammation. The future for SIRT7 is sure to be exciting!

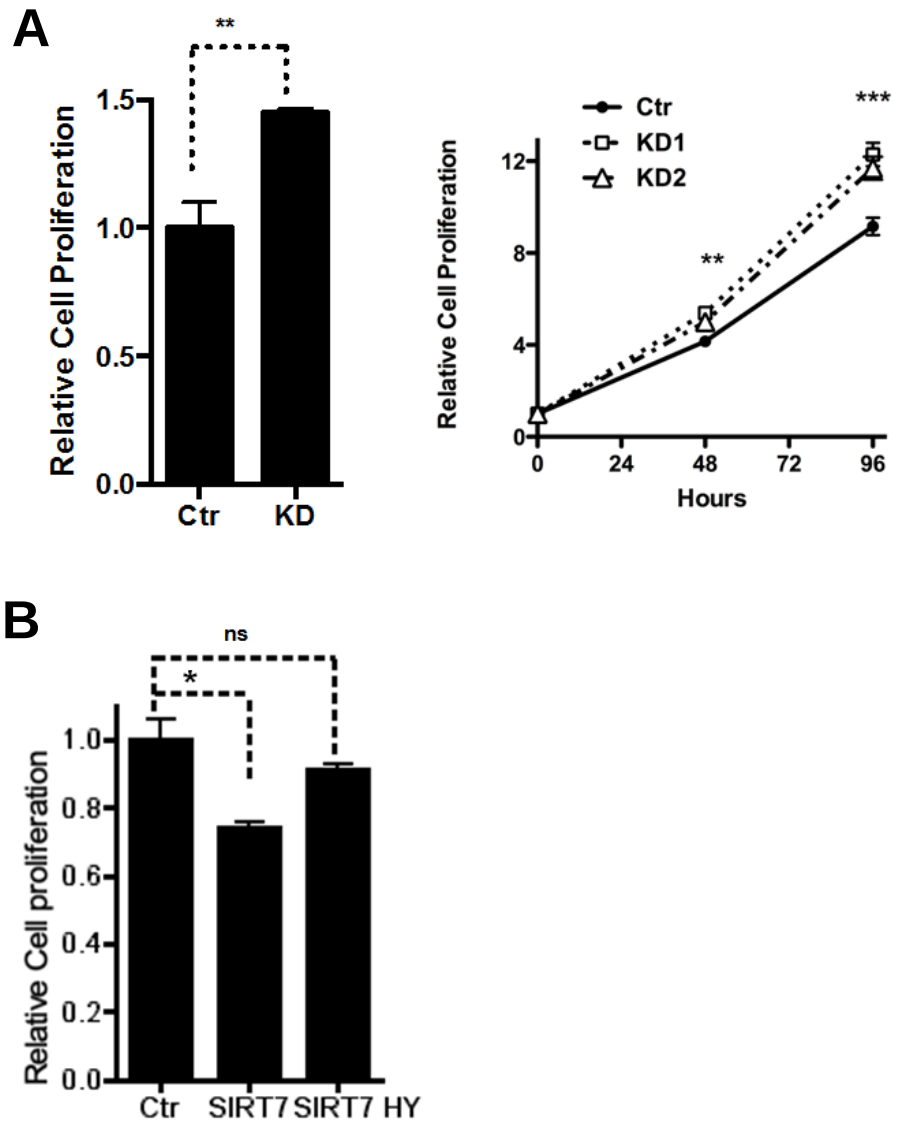


Fig. 4.1: SIRT7 Inhibits Cell Proliferation
(A-B) Increased proliferation in stable SIRT7 KD 293T (A) and HepG2 (B) cells compared to control cells.
(C) Stable SIRT7 overexpressing, but not catalytically inactive SIRT7 (HY), HepG2 cells have reduced proliferation compared to control cells.

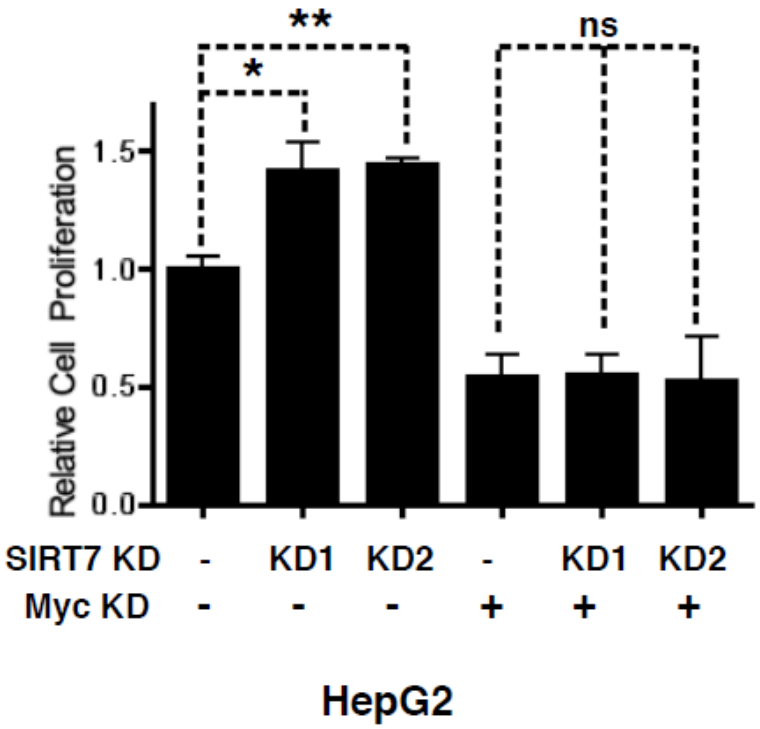


Fig. 4.2: Increased proliferation in SIRT7-deficient cells is Myc dependent. Myc KD attenuates the increased proliferation in stable SIRT7 KD HepG2 cells.

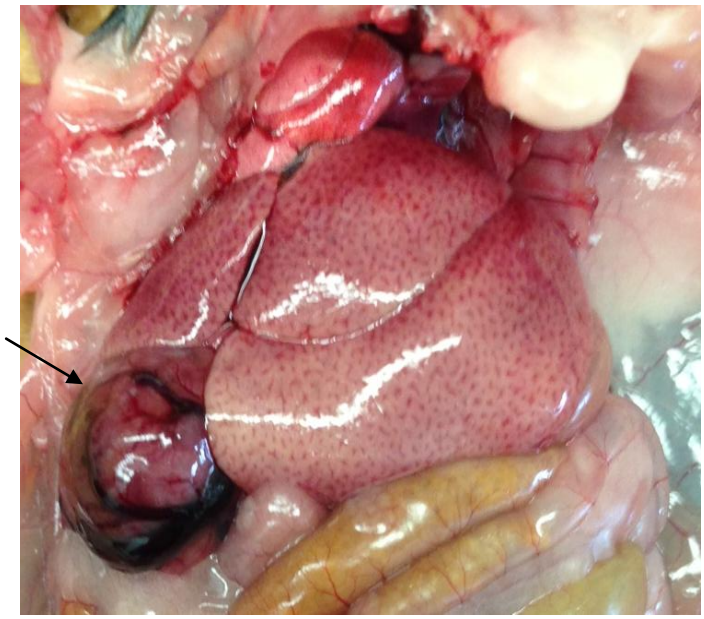


Fig. 4.3: Tumor on liver of 12 month-old SIRT7 KO mouse
Representative photograph of a tumor on the liver of a 12-month old SIRT7 KO mouse.

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Appendix: Materials and Methods

Cell Culture

293T cells, HepG2 cells, and Hepa1-6 cells were acquired from the ATCC. SIRT7 WT and KO MEF cells were generated according to a published protocol (Greber *et al.*, 2007). XBP1 KO MEF were a gift from the Laurie Glimcher lab at Weill-Cornell (Lee *et al.* 2008). Cells were cultured in high glucose DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen). For ER stress induction, cells were treated with 1 ug/mL tunicamycin (Sigma) or 0.1 uM thapsigargin (Sigma) for 24 or 48 hrs. Myc inhibition was performed by treating cells with 2uM 10058-F4 (Sigma) for 24 hours. For mitochondrial stress induction, cells were treated with doxycycline (30 ug/mL) for 48 hrs, ethidium bromide (50 ng/mL) for 7 days, and paraquat (0-400 uM) for 48 hrs. Alternatively, cells were transfected with a construct expressing an aggregation prone mutant mitochondrial OTC protein from the Hoogenraad lab (Zhao *et al.*, 2002) For nutrient deprivation, cells were cultured in glucose free medium (Invitrogen) supplemented with 10% FBS (Invitrogen) or glutamine free medium (Invitrogen) supplemented with 10% FBS (Invitrogen) for 48 hours. Cell proliferation and survival were scored using a Vi-Cell Cell Viability Analyzer (Beckman Coulter). Generation of SIRT7 knockdown and overexpressing cells was described previously (Shin *et al.*, 2013).

RNAi

SIRT7 knockdown target sequences are as follows, as previously described:
S7KD1, 5'-CACCTTTCTGTGAGAACGGAA-3';
S7KD2, 5'-TAGCCATTTGTCCTTGAGGAA-3',
Myc knockdown target sequence is as follows:
Myc KD, 5'- GGACTATCCTGCTGCCAAG -3',
NRF1 knockdown target sequences are as follows:
NRF1 KD (mouse), 5'-GAAAGCTGCAAGCCTATCT-3'
NRF1 KD (human), 5'-CACCGTTGCCCAAGTGAATTA-3'
ATF5 shRNA was a gift from Cole Hayne's lab at Memorial Sloan-Kettering
Double-stranded siRNAs were purchased from Qiagen and were transfected into cells via RNAiMax (Invitrogen) according to manufacturer's instructions.

Co-immunoprecipitations and Immunoblots

Co-immunoprecipitations (Co-IPs) were performed as previously described (Qiu *et al.*, 2010) with Flag-resin (Sigma) or Protein A/G beads (Santa Cruz) for SIRT7 IP. Elution was performed with either Flag peptide (Sigma) or 100mM Glycine solution (pH 3) for SIRT7 IP. Protein samples are boiled at 95C for 10 min before loading. Immunoblotting was performed on an SDS-polyacrylamide resolving gel followed by transfer to nitrocellulose membrane. Membranes were blocked for 30 min in PBS-T with 5% milk. Primary antibody incubation was performed overnight

at 4C. Secondary antibodies incubations were done for 30 min at room temperature. 3x 5min washes with PBS-T were performed between primary and secondary antibody incubations and after secondary antibody incubation. Antibodies for immunoblotting are provided in Table 1.

Polysome Profiling

MEFs or liver tissues were collected for polysomal profiling as described (Zid *et al.*, 2009). Briefly, ten million MEF cells or 0.1 g of liver were harvested and homogenized on ice in 400 ml of solublization buffer (300 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM MgCl2, 1 mM EGTA, 200 mg/ml heparin, 1 mM DTT, 400 U/ml RNAsin plus (Promega), 1X complete, Mini Protease Inhibitor Cocktail (Roche), 0.2 mg/ml cycloheximide, 1% Triton X-100, and 0.1% aodium deoxycholate). Additional solubilization buffer was added for a total of 1ml. Cell lysate was placed back on ice for 10 min before centrifuging at 16,000 g for 15 min at 4C. The supernatant was applied to the top of a 10%–50% continuous sucrose gradient in high salt resolving buffer (140 mM NaCl, 25 mM Tris-HCl [pH 8.0], and 10 mM MgCl2) and centrifuged in a Beckman SW41Ti rotor (Beckman Coulter) at 180,000 g for 90 min at 4C. Gradients were fractionated with continuous monitoring of absorbance at 254 nm.

ChIP and mRNA analysis

Cells were prepared for ChIP as previously described (Dahl and Collas 2007), with the exception that DNA was washed and eluted using a PCR purification kit (Qiagen) rather than by phenol-chloroform extraction. RNA was isolated from cells or tissue using Trizol reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). cDNA was generated using the qScript™ cDNA SuperMix (Quanta Biosciences). Gene expression was determined by real time PCR using Eva qPCR SuperMix kit (BioChain Institute) on an ABI StepOnePlus system. All data were normalized to ActB or GAPDH expression. PCR primers for gene expression are listed in Table 2. PCR primers for ChIP are listed in Table 3.

Flow Cytometry and Cell Sorting

Bone marrow cells were obtained by crushing the long bones with sterile PBS without calcium and magnesium supplemented with 2% FBS. Lineage staining contained a cocktail of biotinylated anti-mouse antibodies to Mac-1a (CD11b), Gr-1 (Ly-6G/C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), and B220 (CD45R) (BioLegend). For detection or sorting, we used streptavidin conjugated to APC-Cy7, c-Kit-APC, Sca-1-Pacific blue, CD48-FITC, and CD150-PE (BioLegend). For congenic strain discrimination, anti-CD45.1 PerCP and anti-CD45.2 PE-Cy7 antibodies (BioLegend) were used. For assessment of cell cycle, Ki-67 (BioLegend) staining was performed according to the manufacturer's recommendation after cell surface staining. For mitochondrial mass, bone

marrow cells were incubated with 100nM MitoTracker Green (Invitrogen) for 30 min at 37°C in the dark after cell surface staining. Dead cells stained by adding 7AAD to cells and incubating for 30 min at 37°C in the dark. All data were collected on a Fortessa (Becton Dickinson), and data analysis was performed with FlowJo (TreeStar). For cell sorting, lineage depletion was performed according to the manufacturer's instructions (Miltenyi Biotec). Cells were sorted using a Cytopeia INFLUX Sorter (Becton Dickinson). Antibody details are provided in Table 4.

In-vitro Stimulation of HSCs

To stimulate HSCs to exit quiescence, freshly isolated HSCs were cultured *ex vivo* in IMDM (Invitrogen) supplemented with 5% Stem Cell FBS (Stem Cell Technologies), 1% Penicillin/Streptomycin, Sodium Pyruvate, NEAA, L-glutamine (Invitrogen), and cytokines (IL-3 (10 ng/mL), GMCSF (10 ng/mL), SCF (25 ng/mL), IL-11 (25ng/mL), Flt3L (25 ng/mL), TPO (25 ng/mL) (Peprotech), and EPO (4 U/mL) (R&D) for 48 hours.

Lentiviral Transduction of HSCs

Sorted HSCs were prestimulated for 24 hr in a 96 well U bottom dish in StemSpan SFEM (Stem Cell Technologies) supplemented with 10% FBS (Stem Cell Technologies), 1% Penicillin/Streptomycin (Invitrogen), IL3 (20ng/ml), IL6 (20ng/ml), TPO (50ng/ml), Flt3L (50ng/ml), and SCF (100ng/ml) (Peprotech). SIRT7 was cloned into the pFUGw lentiviral construct. NRF1 shRNA was cloned into pFUGw-H1 lentiviral construct. Lentivirus was produced as described (Qiu *et al.*, 2010), concentrated by centrifugation, and resuspended with supplemented StemSpan SFEM media. The lentiviral media were added to HSCs in a 24 well plate, spinoculated for 90 min at 270G in the presence of 8ug/ml polybrene. This process was repeated 24 hr later with a fresh batch of lentiviral media. After an additional 24 hr, HSCs were collected for gene expression analyses.

Statistical Analysis

The number of mice chosen for each experiment is based on the principle that the minimal number of mice is used to have sufficient statistical power and is comparable to published literature for the same assays performed. No animals were excluded from the analyses. Mice were randomized to groups and analysis of mice and tissue samples were performed by investigators blinded to the treatment of genetic background of the animals. Statistical analysis was performed with Excel (Microsoft) and Prism 5.0 Software (GraphPad Software). Means between two groups were compared with two-tailed, unpaired Student's t-test. Error Bars represent standard errors. In all corresponding figures, * represents $p<0.05$. ** represents $p<0.01$. *** represents $p<0.001$. ns represents $p>0.05$.

Antibodies	Source	Catalog #
SIRT7	Abnova	H00051547
Beta Actin	Sigma	A2066
Flag	Sigma	F1804
IgG	Santa Cruz	SC-2027
RPS20	Abcam	Ab74700
NME1	Sigma	WH0004830M2
NRF-1	Proteintech	12482-1-AP
MRPL24	Proteintech	16224-1-AP
GFM2	Proteintech	16941-1-AP
ClpP	Proteintech	15698-1-AP
HSP60	Cell Signaling	12165S
RPS14	Abcam	Ab50390
p-eIF2α	Invitrogen	923758A
GRP78	Santa Cruz	Sc-166490

Table 1: Antibodies used in this study

Gene	Primer	Sequence
ATF5	Forward	GGCTCCCTATGAGGTCCTTG
	Reverse	GAGGGAGGAGAGCTGTGAAA
SIRT7 (human)	Forward	CGCCAAATACTTGGTCGTCT
	Reverse	CCCTTTCTGAAGCAGTGTCC
ClpP (human)	Forward	CTCTTCCTGCAATCCGAGAG
	Reverse	GGATGTACTGCATCGTGTCG
Hsp10 (human)	Forward	CAGTAGTCGCTGTTGGATCG
	Reverse	TGCCTCCATATTCTGGGAGA
Hsp60 (human)	Forward	TGACCCAACAAAGGTTGTGA
	Reverse	CATACCACCTCCCATTCCAC
mtDnaJ(human)	Forward	CGAAATGGCAGAAGAAGAGG
	Reverse	TGCATGCACTACAGAGCACA
Grp78 (human)	Forward	TCATCGGACGCACTTGGAA
	Reverse	CAACCACCTTGAATGGCAAGA
ClpP (mouse)	Forward	CTGCCCAATTCCAGAATCAT
	Reverse	TGTAGGCTCTGCTTGGTGTG
Hsp10 (mouse)	Forward	CCAAAGGTGGCATTATGCTT
	Reverse	TGACAGGCTCAATCTCTCCA
Hsp60 (mouse)	Forward	ACCTGTGACAACCCCTGAAG
	Reverse	TGACACCCTTTCTTCCAACC
mtDnaJ (mouse)	Forward	GAGCTGAAGAAGGCATACCG
	Reverse	CAGCTCTCGCTTCTCTGGAT
SIRT7 (mouse)	Forward	CCATGGGAAGTGTGATGATG
	Reverse	TCCTACTGTGGCTGCCTTCT
MRPL16	Forward	ACATACGGGGACCTTCCACT
	Reverse	AAACATGTTCTTGGGGTCCA
MRPL20	Forward	GAACATGAGGACCCTCTGGA
	Reverse	CCGCTAGGACTTTCCTGTTG
MRPL24	Forward	GGGGAACCATGATCCCTAGT
	Reverse	AATTCTCCCTGATCGTGTGG
MRPS31	Forward	GAGGAAGAGTCAAGGGCACA
	Reverse	CTGAATCCGAAGCTCTGGTC
MRPS33	Forward	ATATGCCTTCCGCATGTCTC
	Reverse	GCCAAGGGCAGTTCACTAAA
CHOP	Forward	CCACCACACCTGAAAGCAGAA
	Reverse	AGGTGAAAGGCAGGGACTCA
Erdj4	Forward	CCCCAGTGTCAAACTGTACCAG
	Reverse	AGCGTTTCCAATTTTCCATAAATT
Gadd34	Forward	GAGGGACGCCCACAACTTC
	Reverse	TTACCAGAGACAGGGGTAGGT
PDI	Forward	CAAGATCAAGCCCCACCTGAT
	Reverse	AGTTGCCCCAACCAGTACTT
XBP1s	Forward	GAGTCCGCAGCAGGTG
	Reverse	GTGTCAGAGTCCATGGGA

Table 2: qRT-PCR primer sequences used in this study

Target	Primer	Sequence
□-tubulin	Forward	ACGGGTTTCATCATGTTTGTT
	Reverse	GGCAGATCCCCTGAGGTC
RPS20	Forward	AAGTTCTTTCTTTTTGAGGAAGACG
	Reverse	GAACAGCGGTGAGTCAGGA
GFM2	Forward	CGGGACAGGAAAGAGTCACC
	Reverse	CGGAAAACAGAGGCTCGGAA
mRPL24	Forward	TGAACAGGAAGCCACAACCA
	Reverse	GAGGCCGCTGGGAATTGTAG
NME1	Forward	CCGTAATACTTGGCTCTCGAA
	Reverse	GAATAGACCTGCATGAAGTGAGG

Table 3: ChIP primer sequences used in this study

FACS Antibodies & Reagents	Source	Catalog #	Clone #
Streptavidin PerCP	Biolegend	405213	
Sca1 Pacific Blue	Biolegend	108120	D7
Streptavidin APC-Cy7	Biolegend	405208	
c-Kit APC-Cy7	Biolegend	105826	2B8
CD150 Cy7-PE	Biolegend	115914	TC15-12F12.2
CD3 Biotin	Biolegend	100304	145-2C11
B220 Biotin	Biolegend	103204	RA3-6B2
Gr1 Biotin	Biolegend	108404	RB6-8C5
CD8a Biotin	Biolegend	100704	53-6.8
Mac1 Biotin	Biolegend	101204	M1/70
Ter119 Biotin	Biolegend	116204	TER-119
CD4 Biotin	Biolegend	100404	GK1.5
CD48 FITC	Biolegend	103404	HM48-1
CD150 PE	Biolegend	115904	TC15-12F12.2
c-Kit APC	Biolegend	105812	2B8
Ki67 APC	Biolegend	350514	Ki-67
Fixation buffer	Biolegend	420801	
Permeabilization wash buffer	Biolegend	421002	
7AAD	Biolegend	420404	

Table 4: Flow cytometry antibodies used in this study